Universidade de Aveiro Departamento de Biologia
2014

Nádia Isabel Almeida Osório

O resistoma de *Aeromonas salmonicida* revelado por proteómica

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Professor Doutor António Carlos Matias Correia, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro, da Doutora Ana Sofia Direito dos Santos Duarte, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro e da Doutora Isabel da Silva Henriques, Investigadora Auxiliar do Instituto de Biomedicina da Universidade de Aveiro.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

Referência da bolsa:
SFRH/BD/64343/2009

To my parents and brother

o júri

presidente

Prof. Doutor José Rodrigues Ferreira da Rocha
Professor Catedrático do Departamento de Eletrónica, Telecomunicações e Informática da Universidade de Aveiro

Prof. Doutora Marlene Maria Tourais de Barros
Professora Associada com Agregação do Departamento das Ciências da Saúde da Universidade Católica Portuguesa

Prof. Doutora Gabriela Conceição Duarte Jorge da Silva
Professora Auxiliar da Faculdade de Farmácia da Universidade de Coimbra

Doutor Bruno José Fernandes Oliveira Manadas
Investigador em Pós-Doutoramento do *Conway Institute*, *University Colledge of* Dublin, Biocant e Centro de Neurociências e Biologia Celular da Universidade de Coimbra

Prof. Doutora Ana Cristina Fraga Esteves
Professora Auxiliar Convidada do Departamento de Biologia & CESAM da Universidade de Aveiro

Prof. Doutor António Carlos Matias Correia
Professor Catedrático do Departamento de Biologia da Universidade de Aveiro

agradecimentos

It is a great pleasure to express my deepest gratitude to my supervisors, Professor António Correia, Doctor Ana Sofia Duarte and Doctor Isabel Henriques for introducing to me the fascinating world of microbiology, antibiotic resistance and proteomics.

I'm thankful to Professor António Correia, whose expertise and knowledge helped me to grow up in science.

I'm forever thankful to Doctor Ana Sofia Duarte, Cristina Esteves and Isabel Henriques for their valuable advice during the long laboratory journeys. I will always remember their support and guidance throughout the work, as well as the enthusiastic ideas and new insights and goals to pursue. Thanks for all valuable tips and tricks to overcome laboratory difficulties.

I would like to acknowledge all the people from the MicroLab during all these years for the amazing environment and the good moments.

I owe my warm thanks to colleagues from MicroLab, as Eliana, Dânia, Isabel and Susana. I'm grateful for all support and unconditional help.

I would like to thank to Prof. Bart Devreese, for the lab facilities on proteomics technologies. To Isaak and Laurence a very special thanks for your support in laboratory methodologies.

I would like to acknowledge all members of College of Health Technology of Coimbra, specially my colleagues from Department of Biomedical Laboratory Sciences: Armando Caseiro, António Gabriel, Fernando Mendes, Henrique Santos and Ana Valado. Thanks for support and motivation. I also like to thank the inspiration transmitted by my students.

To Catarina, Rui, thank you for your friendship and for has been my adoptive family during last years. Equally to Patrícia but still with a special thank for patience, the friendly shoulder and all your unconditional support.

I also would like to thank to my dear friends, Susana Gaspar, João, Ana Grácio, Sylvia that have accompanied me during these years, encouraging me and supporting me.

And last but not least, I would like to thank my family (grandparents, uncles and cousins) but especially my parents and my brother Sandro for their support and encouragement, by understanding my prolonged absences and missing important dates. Thank you for always being there when I need!

The most special thanks to my boyfriend for his endless love, support, encouragement and understanding through all these years.

palavras-chave *Aeromonas*; Patogénico oportunista; β-lactâmicos; Resistoma; Proteómica

resumo

Compreender a resposta bacteriana a β-lactâmicos e conhecer os mecanismos de resistência envolvidos é de extrema importância para o tratamento de infeções. Cada vez são mais reportadas as falhas no tratamento de infeções, principalmente decorrentes da expressão de mecanismos de resistência bacterianos. Este estudo teve como principal objetivo obter uma visão global da resposta de *Aeromonas salmonicida* a β-lactâmicos, nomeadamente ampicilina. Focámo-nos nas alterações de expressão das β-lactamases cromossomais indutíveis e nas alterações metabólicas gerais que contribuem para a resistência, numa tentativa de decifrar alguns dos mistérios respeitantes ao "resistoma" desta espécie. Induzimos resistência *in vitro* para ampicilina em *A. salmonicida* CECT894^{T} (As^{WT}) obtendo uma estirpe resistente (As^{R}). Primeiramente fomos avaliar as mudanças nas características fenotípicas de ambas as estirpes quando desafiadas por concentrações subinibitórias de antibiótico. Encontrámos uma redução na taxa de crescimento, um aumento na expressão de β-lactamases e um aumento na capacidade de formar biofilme mostrando que esses processos detêm extrema importância na resposta à presença do antibiótico. Porém a produção de fatores de virulência como hemolisinas, proteases e toxinas não mostrou estar relacionada diretamente com o desafio de antibiótico, mas sim dependente da suscetibilidade inicial da estirpe. Posteriormente, e a fim de conhecermos melhor os determinantes genéticos e os mecanismos envolvidos nas mudanças fisiológicas que ocorrem durante a adaptação ao antibiótico fomos comparar os subproteomas (intra e extracelular) das duas estirpes por eletroforese em gel bidimensional. Detetámos várias proteínas diferencialmente expressas, 119 a nível intracelular e 53 a nível extracelular. Estas proteínas mostraram-se envolvidas em diferentes vias metabólicas proporcionando uma visão global da resposta celular adaptativa aos β-lactâmicos. Como alterações principais verificámos um aumento na expressão de β-lactamases, uma diminuição na permeabilidade de membrana, uma sobreexpressão de bombas de efluxo, uma diminuição da taxa de divisão celular, alterações metabólicas várias (aumento da síntese de proteínas, produção de energia e catabolismo de hidratos de carbono) e aumento da resposta a *stress* oxidativo. Em conclusão, *A. salmonicida* responde rapidamente a β-lactâmicos adaptando os seus mecanismos intrínsecos de resistência e promovendo simultaneamente uma resposta que envolve um fenótipo de "persistência", crescimento bacteriano em biofilme, resposta SOS com eventual hipermutação. Adicionalmente a deteção de β-lactamases no meio extracelular sugere um possível envolvimento de vesículas membranares como mecanismo de defesa.

resumo
(continuação)

Este estudo torna-se o primeiro a recorrer a proteómica para avaliar a resistência a β-lactâmicos numa espécie de Aeromonas dando indicações preciosas sobre determinantes genéticos relacionados com o “resistoma”, que poderão ser cruciais para o futuro desenvolvimento de novas terapêuticas.

keywords *Aeromonas*; Opportunistic pathogen; β-lactam; Resistome; Proteomics

abstract

Understand the β-lactam response and resistance mechanisms in bacteria is of great value. The increasing failure to treat bacterial infections is maily due to the expression of resistance mechanisms. The aim of this study was to assess the response of *Aeromonas salmonicida* to β-lactams, mainly ampicillin. We focused on induced and chromosomally located β-lactamases, as well as on the general metabolic changes induced by the presence of the antibiotic, in an attempt to unveil some of the mysteries of the β-lactam "resistome" in this species. Resistance to ampicllin was induced experimentally in *A. salmonicida* CECT894^{T} (As^{WT}), creating a derivate ampicillin resistant strain (As^{R}). First, we evaluated changes that occur at phenotypic level in both strains when challenged by sub inhibitory concentrations of the antibiotic. We found a decrease of growth rate, an increase in expression of inducible β-lactamases and an increase of the ability to form biofilm; these processes must be of major importance for antibiotic stress response. However, the production of virulence factors as haemolysins, proteases and toxins, showed not to be related to the antibiotic challenge but to the initial antibiotic susceptibility phenotype of each strain. Afterwards, in order to get more insights into the physiological changes upon antibiotic stress we compared subproteomes (intra and extracellular) of both strains using two-dimensional gel electrophoresis. The intracellular proteome revealed 119 proteins with changes in abundance and extracellular proteome revealed 53 proteins. Identification of these proteins and the pathways in which they are involved provided a global view of cell metabolism related to antibiotic resistance. We observed as principal changes, an increase in β-lactamase production, a decrease in membrane permeability, overexpression of efflux pumps, a decrease of cell division (growth rate reduction), shifts in metabolism (increase in proteins biosynthesis, in energy production, catabolism of carbohydrates) and an increase in oxidative stress in consequence of an adaptive response to β-lactam antibiotic. In conclusion, we found that *A. salmonicida* quickly responds to a β-lactam challenge, activating their intrinsic mechanisms of resistance and concurrently producing an adaptive response involving different processes, such as "persister" phenotype (reduced growth), growth in biofilm-mode, SOS response with eventual hypermutation. We also postulate the possible involvement of outer membrane vesicles as a defense mechanism. This work is the first proteomic study of antibiotic challenge in a *Aeromonas* spp., gaining insights into the β-lactam "resistome". The identification of the components of this "resistome" is useful for the future development of new therapies.

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IV – ABBREVIATIONS

AHM-PP	1,6-anhydromuramyl pentapeptide
AMP	Ampicillin
ARGs	Antibiotic resistance genes
CHAPS	Cholamidopropyl dimethylammonio-1-propanesulfonate
CLSI	Clinical and Laboratory Standards Institute
CFU	Colony forming unit
DIGE	Difference gel electrophoresis
M5	Disaccharide-pentapeptide monomer
DTT	Dithiothreitol
EP	Efflux pumps
ESBL	Extended-spectrum β-lactamase
ECP	extracellular products
GO	Gene ontology
IPG	Immobilized pH gradient
IEF	Isoelectric focusing
LPS	Lipopolysaccharide
LC MS-MS	Liquid chromatography–mass spectrometry
LB	Luria Broth
MS	Mass spectrometry
MALDI	Matrix-assisted laser desorption/ionization
MIC	Minimum inhibitory concentration
MHA	Muller Hinton Agar
AHL	N-acylhomoserine lactones
OD	Optical density
OM	Outer membrane
OMP	Outer membrane proteins
PBP	Penicillin-binding proteins
PG	Peptidoglycan
QS	Quorum sensing
ROS	Reactive oxygen species

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TOF-TOF	Tandem mass spectrometry
TA	Toxin-antitoxin
TCA	Tricarboxylic acid
TSA	Tryptic Soy Agar
2D	Two dimensional
TCR	Two-component Regulator

CHAPTER I

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1. THE GENUS *AEROMONAS:* GENERAL OVERVIEW

The genus *Aeromonas* belongs to the family *Aeromonadaceae* and its taxonomy at the species level is complex (Janda & Abbott 2010). Members of this genus are widely distributed in the environment. They are able to inhabit natural soil, they appear in food products and are hosted by several animals, but most commonly occur in all kinds of aquatic environments.

Aeromonas species are distributed in two groups: psychrophilic and mesophilic bacteria. The psychrophilic group contains nonmotile bacteria with growth temperature between 22-25ºC and that are the cause of variable diseases in fish (Dallaire-Dufresne et al. 2014). In contrast, the mesophilic group contains motile bacteria with optimum growth temperature between 35-37ºC some of which are considered opportunistic human pathogens (Parker & Shaw 2011).

1.1.1. General characteristics of the *Aeromonas* genus

Aeromonas are Gram negative rods, facultative anaerobes which are cytochrome oxidase, catalase and indole positive (Parker & Shaw 2011). In several species isolated from humans, it has been found ≈90% of the strains producing β-hemolysis (Janda & Abbott 2010). They are able to ferment maltose, D-galactose and trehalose and unable to ferment xylose, sorbose, erythritol, adonitol, dulcitol (Parker & Shaw 2011).

Members of this genus often produce several extracellular hydrolytic enzymes, such as amylase, deoxyribonuclease, elastase and lipase. They grow at a wide range of temperature, from 0ºC to 45ºC, but the optimum range is 22ºC to 35ºC. Normally, they also grow well in alkaline pH, with an optimum range between 5.5-9.0 (Ghenghesh et al. 2008). *Aeromonas* from the mesophilic group normally possess a single polar flagella (Parker & Shaw 2011).

1.1.2. Diversity and distribution of *Aeromonas* species

According to recent data, genus *Aeromonas* is composed by 25 species (Beaz-Hidalgo & Figueras 2013), distributed into two main groups: the psychrophilic and mesophilic bacteria. Within the mesophilic group stand out *Aeromonas hydrophila, Aeromonas caviae, Aeromonas jandaei and Aeromonas veronii* bv. *sobria* which are responsible for a great number of infections in humans and other animals (Janda & Abbott 2010). These species are widely distributed in nature, being mainly present in aquatic environments (rivers, lakes, estuaries, wastewaters, aquacultures and urban drinking water), food (milk, vegetables and meat) and animals (cat, dogs, cows, fish and prawns) (Ghenghesh et al. 2008). Within the psychrophilic group stands out *Aeromonas salmonicida,* responsible for a great number of fish infections particularly in salmonids (Dallaire-Dufresne et al. 2014). This species is present in aquatic environments (Beaz-Hidalgo & Figueras 2013) (Figure1).

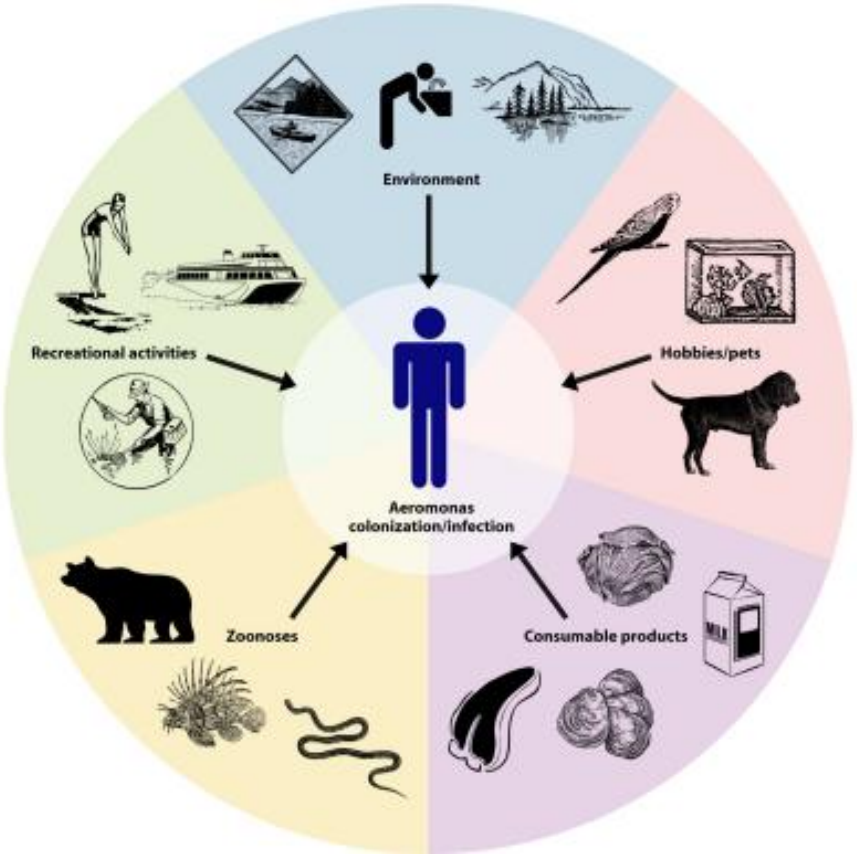

Figure 1. Environmental sources of *Aeromonas* species with potential for human infection/colonisation (Janda & Abbott 2010).

1.2. THE PATHOGENIC POTENTIAL OF *AEROMONAS* SPP.

Aeromonas species are present in nature and easily become opportunistic pathogens in fish and humans, causing infections in debilitated organisms. The infection process is mainly dependent on the expression of virulence mechanisms by the bacteria. *Aeromonas* species are able to produce several virulence factors namely adhesins and exoenzymes (Merino et al. 1995). All these features are dependent of quorum sensing (cell to cell communication).

The number of infections reported to be caused by members of this genus is increasing, with variable success in treatment. Several studies have shown that fluoroquinolones and cefotaxime are the best choices for treatment (Parker & Shaw 2011).

1.2.1. *Aeromonas* spp. as opportunistic pathogens

Aeromonas has emerged as an important pathogen in humans causing a wide variety of infections, namely gastroenteritis as most common disease, wound and soft tissue infections, septicemia, respiratory infections and other infections, often in immunocompromised individuals (Parker & Shaw 2011; Chopra & Houston 1999). Among animals, fishes are the most prone to infection. This opportunistic pathogens cause significant economic losses in aquacultures due to furunculosis (furuncles or boils that develop on the skin and on musculature promoting a sub-acute or chronic disease) and systemic diseases in salmons and trout characterized by high mortality and morbidity (Dallaire-Dufresne et al. 2014; Beaz-Hidalgo & Figueras 2013).

1.2.2. Virulence factors

The bacteria synthesize virulence factors during the infection process and promote several types of interactions between the pathogen and the host. Bacteria can only infect a specific host, if their virulence factors can overcome the defense mechanisms of the host. The main virulence factors of *Aeromonas* spp. are: adhesins, extracellular products, iron acquisition mechanisms, secretion systems and quorum sensing (QS) (Merino et al. 1995).

1.2.2.1. Adhesins

Adhesins are the proteins responsible for the first contact between the host and the pathogen. In *Aeromonas* there are two types of adhesins: filamentous (flagella and fimbriae) and non-filamentous, which include lipopolysaccharide (LPS), capsule and outer membrane proteins (OMP) (Beaz-Hidalgo & Figueras 2013). Within filamentous adhesins, flagella have been described in *Aeromonas* spp. with different morphological types, short, rigid (S/R), which are numerous per cell and long, and flexible (L/W), which are fewer per cell. Both kinds increase the adhesion to different cell lines (Merino et al. 1995). Related to flagella, some species express pili (fimbriae) responsible for host adherence and virulence. *A. salmonicida* was shown to have pilus types I and IV, the type I being recognized as able to promote adherence in the gastrointestinal tract in salmons, and the type IV shown to be related to epithelial adherence, colonization, cellular invasion, formation of biofilm and virulence. The genes encoding this type of pili were also found within the genomes of *A. hydrophila* and *Aeromonas aquarium* (Beaz-Hidalgo & Figueras 2013). Within non filamentous fimbriae, the lipopolysaccharide (LPS) is the structure present in outer membrane (OM) responsible for the inflammatory activity mainly due to the O-polysaccharide (O-antigen). They are associated with the A-layer (OMP), a complex protein structure that increases the hydrophobicity of the bacterial surface and is responsible for bacterial autoagglutination, adherence and resistance to macrophages and also providing protection against complement factors of the host (Beaz-Hidalgo & Figueras 2013; Dallaire-Dufresne et al. 2014). The capsule is a layer of polypeptides and polysaccharides covering the OM and is important for avoidance of the host immune defenses. Bacteria with capsule have been shown to display more ability to adhere and greater resistance to phagocytosis (Merino et al. 1995).

1.2.2.2. Extracellular products

Several species of *Aeromonas* can produce a variety of biologically active extracellular products. These include hemolysins, cytotoxins, enterotoxins, proteases, leukocidin, phospolipases and endotoxins. The main goal of these products is to allow the invasion of host cells, tissues and dissemination of

infection, does contributing to the pathogenesis (Chopra & Houston 1999). Proteases (as serine proteases, metalloproteinases and collagenase) are regarded as major virulence factors, as is the case of a serine protease (AspA) in *A. salmonicida* that liquefies the muscle tissues, causing furunculosis. This protein is closely associated to a lipase (SatA) which hydrolyzes membrane phospholipids promoting cell lysis (Dallaire-Dufresne et al. 2014). Enterotoxins in turn play an important role in intestinal disease. For example *A. hydrophila* produces two types of enterotoxins that cause a fluid secretion (Chopra & Houston 1999). Cytotoxins include hemolytic, cytotoxic and enterotoxin activities and their main target are the host cells (epithelial and erythrocytes cells). An example is the Act toxin purified in *A. hydrophila* SSU that shows a behaviour of hemolysin, closely related to AerA, an aerolysin present in many others species (Chopra & Houston 1999).

1.2.2.3. Iron acquisition mechanisms

Sequestration of iron inside the host cells is an essential mechanism for surveillance of most bacteria within the host. Several bacterial mechanisms contribute their virulence by inhibiting the defense action of the host's transferrin and enhancing the resistance to the bactericidal activity of the host phagocytes. The mechanisms to obtain iron in *Aeromonas* spp. can be siderophore dependent or independent (Beaz-Hidalgo & Figueras 2013). Siderophores are low molecular weight iron chelators that remove iron from the host binding proteins, allowing it to enter the bacterial cell through OM receptor proteins. *Aeromonas* species produce siderophores such as enterobactin and amonobactin (Chopra & Houston 1999) and *A. salmonicida* is also able to synthesize an anguibactin-like siderophore (Beaz-Hidalgo & Figueras 2013). Although siderophores bind iron with high affinity, they cannot remove iron from hemoglobin. This can be achieved by siderophore independent systems as heme binding proteins detected in *A. salmonicida* (Ebanks et al. 2004).

1.2.2.4. Secretion systems

The secretion systems consist in a transmembrane injection apparatus composed of integral membrane proteins and a needle-like structure to

translocate a range of effector proteins from the bacteria cytosol directly into host cells. In *Aeromonas* spp. were described four types of secretion systems, type II (T2SS), III (T3SS), IV (T4SS) and VI (T6SS). The T2SS is responsible for secretion of toxins and degradation enzymes, for example aerolysins, DNAses and proteases (Vanderlinde et al. 2014). The T3SS, referred as "injectisome" is considered the most important virulence secretion system in *A. salmonicida.* This is because the effector proteins normally disrupt the host cell cytoskeleton and induce apoptosis in B and T lymphocytes, thereby promoting a reduction in the host immune response, an important feature in fish disease (Bergh & Frey 2014). The T4SS is similar to other secretion systems, but is the only one capable of performing conjugal genetic transfer between bacteria, therefore being important in the spread of antibiotic resistance genes (ARGs) (Rangrez et al. 2010). The T6SS is able to export effector proteins into the extracellular milieu and/or is able to translocate them into eukaryotic host cell cytoplasm. This system has shown importance in gastrointestinal disease caused by *A. hydrophila*, mainly due to the VgrG1 protein, that has actin ADPRT activity. This protein can trigger actin depolymerization with fatal effects on the intestinal epithelial barrier, thus allowing the entry of other virulence factors associated with pathogenesis (Suarez et al. 2010).

1.2.2.5. Biofilm formation

Bacteria can be present in nature either as single free suspended cells or associated with a surface – a biofilm. In a biofilm, microbial community is attached to a surface and embedded in a self-produced matrix (Burmolle et al. 2006). This structure provides several advantages, namely to maintain bacteria in a selected microenvironment, protecting them from the action of antibiotics, biocides and physical challenges and allowing the development of stress response (Stewart 2002).

The presence of a charged hydrated exopolymer matrix around individual cells and microcolonies, profoundly affects the access of solutes. Thus biofilms result in a lower diffusion of substrates like nutrients and neutralization of reactive molecules like antibiotics. Microenvironments are produced in biofilms due to a differential diffusion of these compounds, allowing heterogeneity in bacterial

population. The slow growing bacteria, called persisters represent perhaps 0.1-10% of all cells living in biofilm and are able to survive for example to a catastrophic antimicrobial challenge, because they can adapt quickly switching metabolic pathways (Roberts & Stewart 2005).

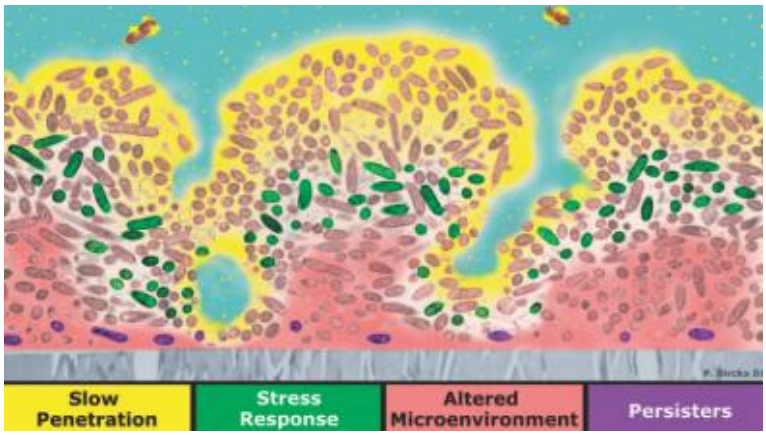

Figure 2. Possible mechanisms of biofilm tolerance to antibiotics: slow antibiotic penetration (yellow); bacterial stress response activation (green); altered microenvironment, nutrient depletion (pink); persisters, protected phenotype (purple) (Chambless et al. 2006).

In addition, the exopolymer matrix also retains the drug inactivating enzymes when produced by some bacterial cells inhibiting the effect of antibiotic. Hence, survival of biofilm communities must relate to the adoption or clonal expansion of resistant phenotype (Gilbert et al. 2002). Therefore, a biofilm presents higher cells density and higher levels of cellular communication, contributing to increase not only the antibiotic resistance but also virulence through the QS signaling. Biofilm formation play an important role in persistence of several infections (Mah & O'Toole 2001).

1.2.2.6. Quorum-sensing (cell-to-cell communication)

QS is a bacterial intercellular communication system that enables synchronization of gene expression in the establishment of phenotypes like virulence, biofilm formation and antibiotic resistance. The growth of bacteria is accompanied by the release of small molecular mass signal molecules into environment, called N-acylhomoserine lactones (AHL). When these molecules

achieve a threshold of concentration, bacterial population responds with changes in expression of some genes. The AHL molecules are recognized by specific bacterial cell receptors that belong to a large class of DNA binding transcription factors named “R-proteins”, such as LuxR (Li & Nair 2012).

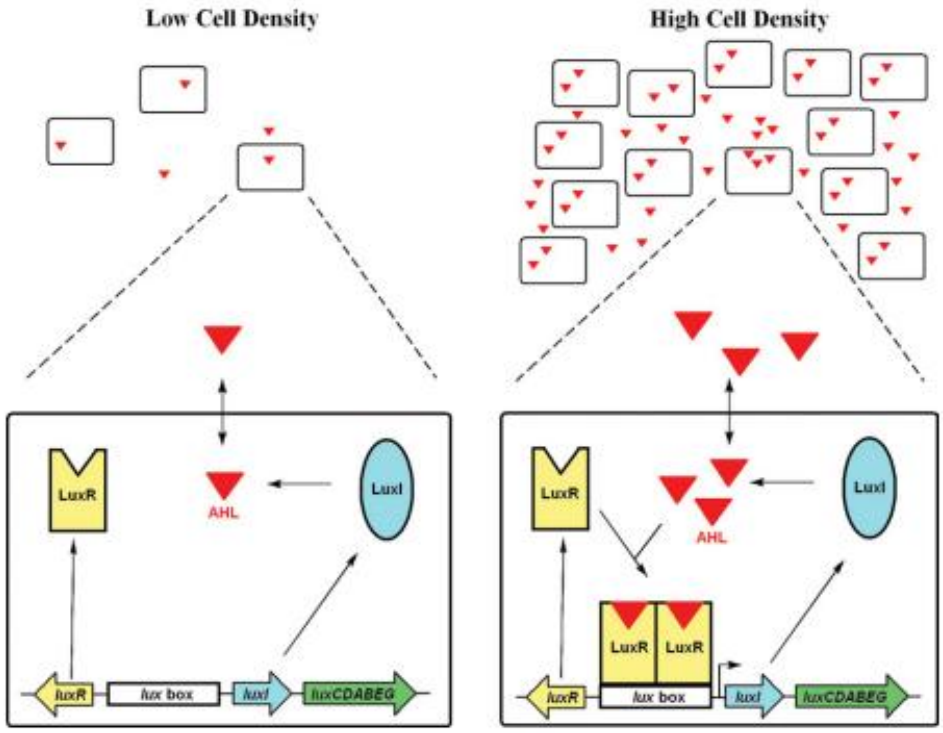

Figure 3. Acylhomoserine lactone (AHL)-dependent quorum sensing system as exemplified by LuxI/R system in *Vibrio fischeri*. AHL, QS-signal molecule (red); LuxR, DNA-binding transcription factor (yellow); LuxI, *luxI* gene that encodes the AHL synthase (blue) (Li & Nair 2012).

1.2.2.6.1. Regulation of QS in *Aeromonas* spp.

QS in *Aeromonas* spp. is mediated by AHL and is responsible for the control of expression of many virulence factors. This genus produces two types of lactones, namely N-3-butanoyl-DL-homoserine lactone (C4-HSL) and N-3-hexanoyl-homoserine lactone (C6-HSL), of which C4-HSL is the predominant type (Khajanchi et al. 2009). AHL are synthesized by the LuxI protein family. It diffuses freely inside and outside bacterial cells and when reaching a certain concentration, it binds receptors known as LuxR proteins which in turn, regulate the transcription process of many genes involved in virulence. LuxI and LuxR

proteins in *A. hydrophila* are known as AhyI and AhyR, respectively; and in *A. salmonicida*, AsaI and AsaR (Swift et al. 1997).

Besides the LuxRI (auto-inducer 1) at least other two QS systems are known in *Aeromonas*: LuxS (auto-inducer 2) and QseBC signaling system (auto-inducer 3).

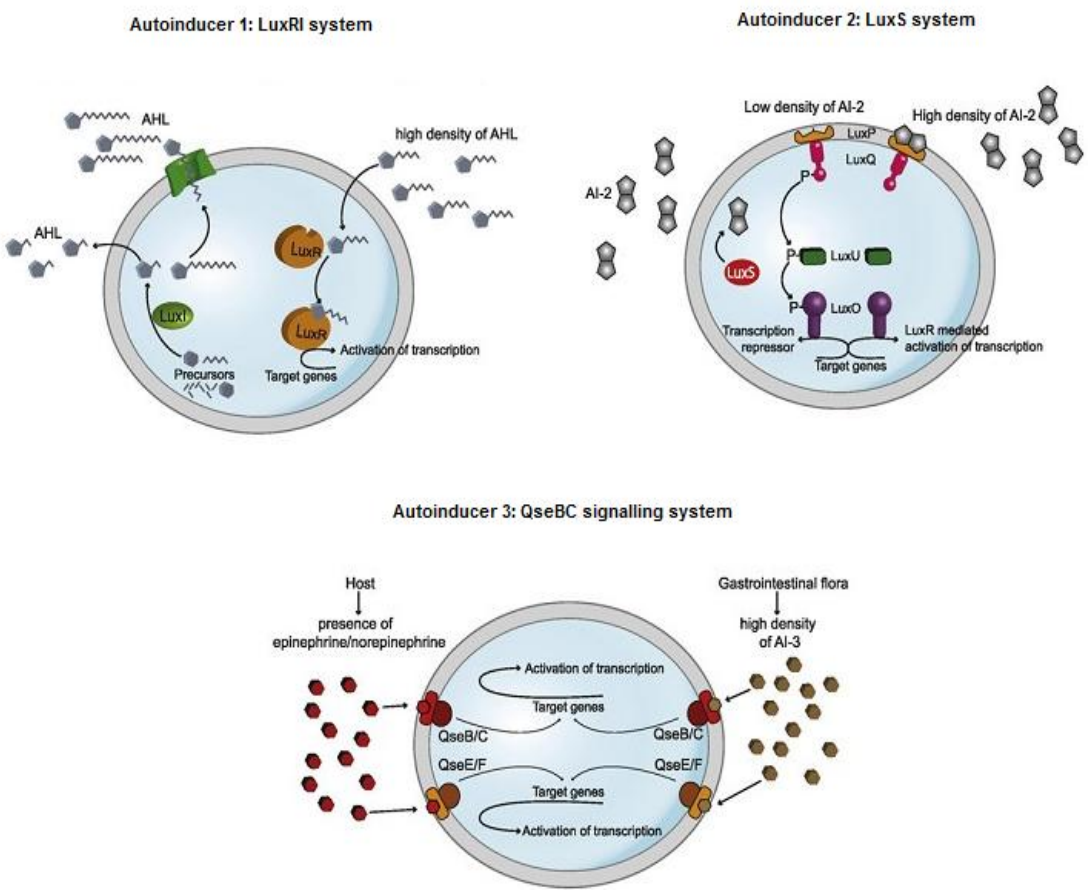

Figure 4. Quorum sensing systems described in *Aeromonas* spp. (adapted from Boyen et al. (2009))

All QS systems coexist in *Aeromonas* spp. and they are regulated by intracellular levels of c-di-GMP (intracellular second messenger). The c-di-GMP levels modulate the activity of response regulators and these in turn regulate the transcription of genes involved in virulence. In *A. hydrophila* SSU, *ahyRI* genes were described as able to positively regulate the bacterial virulence (metalloprotease production, biofilm formation and secretion of the type T6SS effectors), and the LuxS system was described as able to negatively regulate

virulence factors (however, it increases the biofilm formation) and QseBC signalling system was described as able to positively regulate motility, hemolytic activity and protease production while inhibiting the biofilm formation. However all these response regulators are up-regulated by c-di-GMP levels. The intracellular c-di-GMP levels fluctuate according to phosphorylation-dependent signalling pathways (Kozlova et al. 2011; Khajanchi et al. 2012).

1.2.3. Treatment of *Aeromonas* spp. infections

Commonly *Aeromonas* spp. display high resistance rates against penicillins and first generation cephalosporins due to chromosomal β-lactamases production. Hence these antibiotics are not a good choice for treatment of *Aeromonas* infections. Within the different chromosomal β-lactamases of *Aeromonas* spp. we have to highlight the CphA/ImiS enzyme, β-lactamases that have been implicated in carbapenem resistance. Thus, carbapenems are not used to treat *Aeromonas* infections. The most suitable treatment implies the use of fluoroquinolones, such as ciprofloxacin (Parker & Shaw 2011; Ghenghesh et al. 2008).

The members of this genus are mainly present in aquatic environments, which are important reservoirs of ARGs. Thus, these bacteria may acquire resistance to different classes of antibiotics, making treatment more difficult (Piotrowska & Popowska 2014). For example, several *Aeromonas* strains have been shown to contain mobile genetic elements such as integrons which may encode resistance to aminoglycosides, chloramphenicol and trimethoprim (Kadlec et al. 2011; Moura et al. 2007). Thus, *Aeromonas* may be vectors for the spread of ARGs.

1.3. β-LACTAM RESISTANCE

Resistance to β-lactams in *Aeromonas* spp. arises directly associated with the production of chromosomally encoded inducible β-lactamases, recognized as the major mechanism of antibiotic resistance in this genus (Ko et al. 1998). The expression of genes encoding different β-lactamases in the members of this genus is coordinated by a common regulatory pathway and this is activated by antibiotic presence (Alksne et al.1997; Taylor et al. 2010). In this sense, this mechanism is frequently used by *Aeromonas* spp. to express resistance. However, other mechanisms are known to also contribute to the resistance phenotype.

1.3.1. β-Lactam antibiotics

Antibiotics of the β-lactam group share a common core in their structure called the β-lactam ring and can be classified as penicillins, cephalosporins, monobactams and carbapenems.

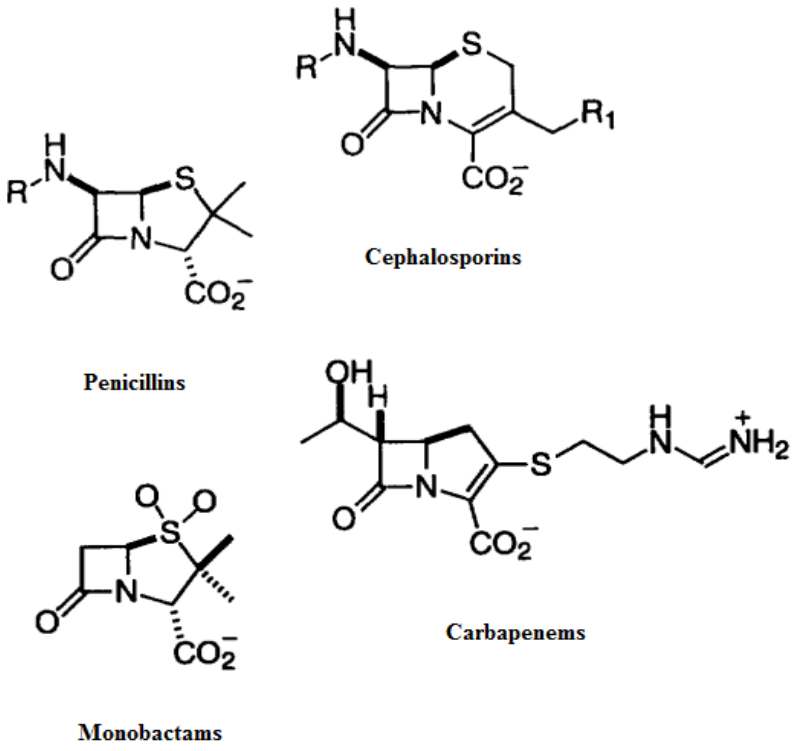

Figure 5. Structure of β-lactam antibiotics (adapted from Kotra & Mobashery (1998)).

1.3.1.1. Mode of action of β-lactam antibiotics

β-lactam antibiotics inhibit the bacterial wall synthesis through the inactivation of the transpeptidation process required for the formation of the peptidoglycan (PG). The PG is a rigid, insoluble heteropolymer responsible for maintaining cell shape and internal osmotic pressure. It consists in glycan strands cross-linked through short peptides. The glycan strands are composed of alternating N-acetylglucosamine and N-acetylmuramic acid saccharides and their polymerization and cross linking of the peptides depend on the activity of penicillin-binding proteins (PBPs). These, proteins are recognized by β-lactams, which inhibit their function stopping PG synthesis (Heijenoort 2001; Johnson et al. 2013).

1.3.2. Intrinsic resistance mechanism to β-lactam antibiotics

The intrinsic resistance is the innate ability of bacterial species to resist to a particular antimicrobial agent through its inherent structural and/or functional characteristics. This can also be called “insensitivity” and can be due to lack of affinity of the drug for the bacterial target, inaccessibility of the drug to the bacterial cell, extrusion of the drug by chromosomally encoded active exports, innate production of enzymes that inactivate the drug. However all these mechanisms may be adapted and/or activated according to the stress caused by the presence of antibiotic.

1.3.2.1. β-lactamases production

The most common mechanism of resistance to β-lactam antibiotics is the ability of bacteria to express β-lactamases. These enzymes render the antibiotic ineffective by hydrolytic cleavage of the β-lactam ring (Kotra & Mobashery 1998). *Aeromonas* can produce various β-lactamases which confer resistance to a broad spectrum of β-lactam antibiotics. Three classes of β-lactamases have been recognized encoded by unlinked genes: class B, metallo-β-lactamases encoded by *imiS/cphA* gene; class C, AmpC β-lactamases encoded by *cep* gene and class D, penicillinase encoded by *amp* gene (Chen et al. 2012; Niumsup et al. 2003).

1.3.2.1.1. Regulation mechanism of β-lactamases expression

In Gram negative bacteria, chromosomal β-lactamases expression is commonly regulated by AmpC/AmpR system. This system explains the expression of a single β-lactamase gene, *ampC* that is controlled negatively and positively by LysR-type transcription factor, AmpR. The activity of AmpR is determined by 1,6-anhydromuramyl pentapeptide (AHM-PP), a product of PG turnover and the substrate for the PG recycling pathway. Normally, this monomer is transported into the cytoplasm by the specific permease, AmpG and does not accumulate because it is broken down by an amidase, AmpD, being reused in murein biosynthsesis. Thus, its levels will regulate AmpR activation, which consequently controls *ampC* expression. Exposure to antibiotics alters the balance in favour of intracellular accumulation of AHM-PP and *ampC* expression is induced (Jacobs et al. 1997).

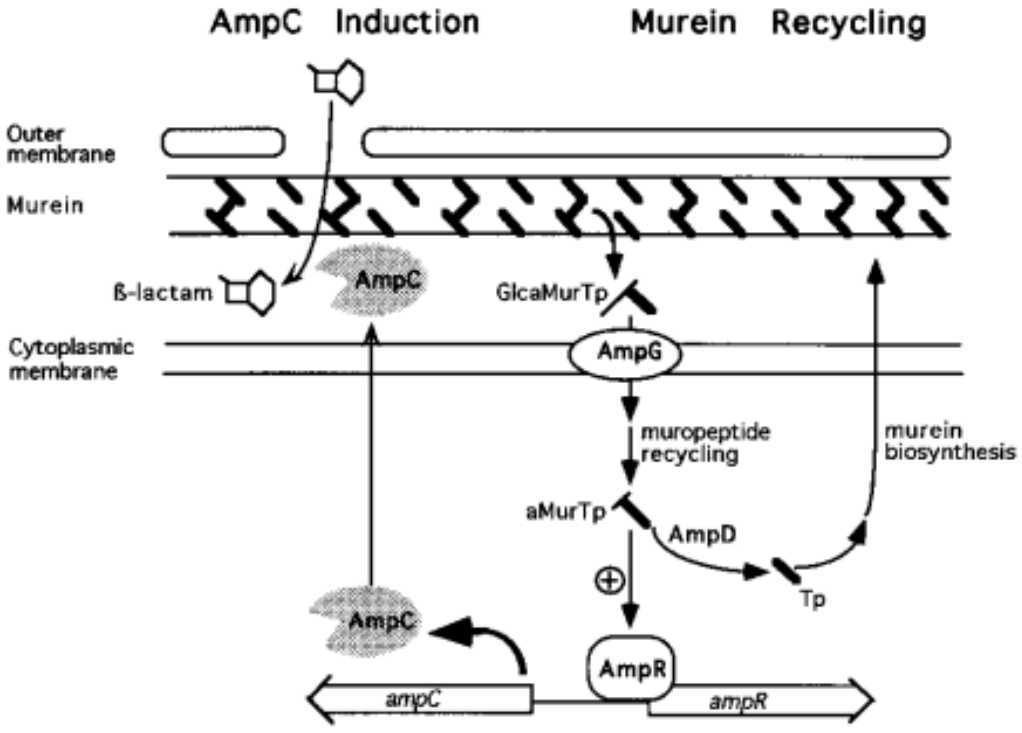

Figure 6. β-lactamase expression regulated by AmpC/AmpR system (Jacobs et al. 1997)

However the coordinated expression of multiple β-lactamases in *Aeromonas* spp. does not involve an AmpR-like regulator but rather two-component

regulator (TCR), called *blrAB* system. BlrA is a transcription factor (phosphorylation-dependent response regulator) able to activate the expression of several β-lactamase genes and BlrB is a sensor kinase (Niumsup et al. 2003). The expression levels of different β-lactamases depend on the number of blr-tag DNA-sequences (DNA binding domains of the transcription factor, BlrA) found upstream the promoters of genes that encode β-lactamases (Avison et al. 2004). Thus, the expression level of AmpH, CepH and ImiH can be different.

The BlrAB induction mechanism is dependent on the β-lactams challenging the cells. It is the disruption of PBP that promotes higher levels of M5 (a disaccharide pentapeptide, component of PG) and this leads to phosphorylation of the periplasmic domain of BlrB producing a response that increases the β-lactamases expression (Tayler et al. 2010).

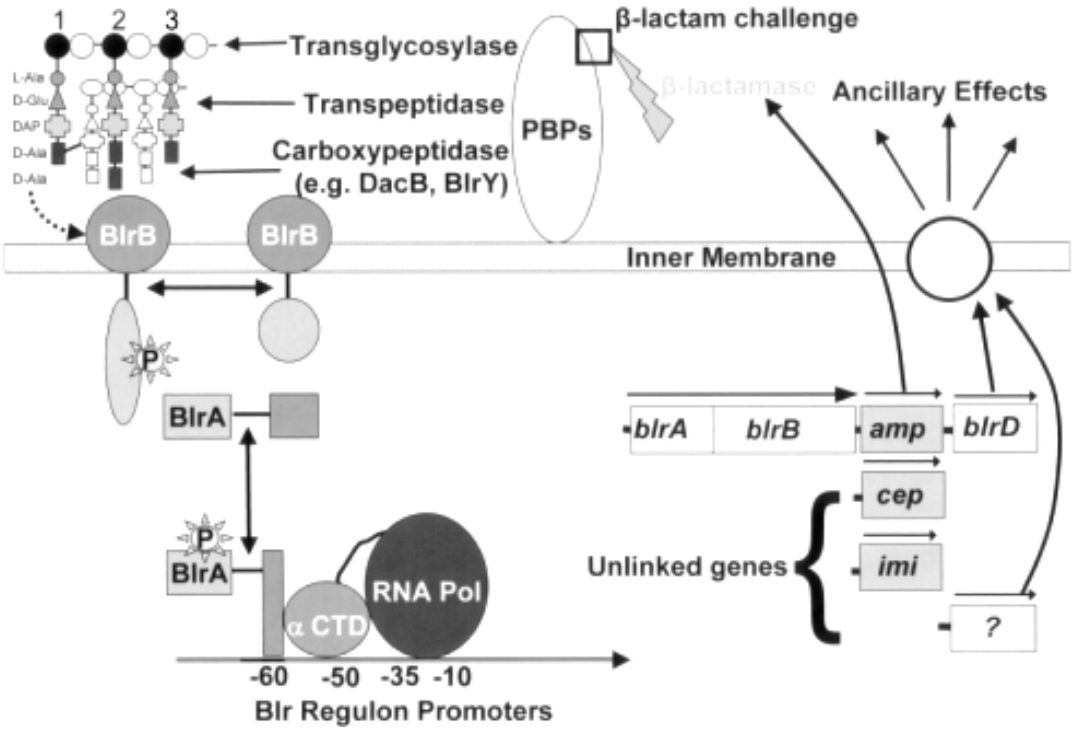

Figure 7. Model for β-lactamases induction in *Aeromonas* spp. and the role of the BlrAB system (Tayler et al. 2010)

1.3.2.2. Membrane permeability

The OM combines a highly hydrophobic lipid bilayer with pore-forming proteins of specific size exclusion properties acting as a selective barrier. The permeability properties of this barrier have a great impact on the susceptibility of microorganisms to antibiotics. Small hydrophilic drugs, such as β-lactams, use the pore-forming porins to gain access to the cell interior, mainly to periplasm (Delcour 2009).

A large number of different types of proteins reside in OM, some of them being extremely abundant like OmpA. In *Aeromonas* spp. OmpA porins are coded by two genes (*ompAI* and *ompAII*), and their main function is to contribute for the selective permeation responsible for processes like osmoregulation, nutrient acquisition and export of proteins or waste products (Ebanks et al. 2005). The expression of these porins is dependent on cell osmolarity and on the presence or absence of AMPc. In other words, changes at this level alter a cytosolic response regulator (OmpR protein) able to modify transcription processes involving genes that encode porins. In *Aeromonas* spp. changes in OMP are directly associated to the loss of exoprotease activity due to lower export of caseinase, the major exoprotease in this genus (Masi & Pagès 2013; Griffiths & Lynch 1989).

1.3.2.2.1. Outer membrane vesicles

Bacterial outer membrane vesicles (OMV) play a key role in innate bacterial defense against environmental stressors that target the cell envelope, for example β-lactam antibiotics. OMV are spherical portions of bacterial envelope containing OMP and lipids as well as soluble material contained in the lumen or bounded to external surface (Manning & Kuehn 2011). They act as potent “communicasomes” allowing an increase in bacterial survival and pathogenesis (Lee & Choi 2008).

OMVs are associated with export of proteins when membrane permeability is modified and, for this reason, may contain a great diversity of proteins related to invasion, adherence, antibiotic resistance, biofilm formation and other virulence factors (Bonnington & Kuehn 2014). In *A. c*aviae, OMV production was related

to biofilm formation showing their importance in cell-to-cell communication (Angeles-morales et al. 2012).

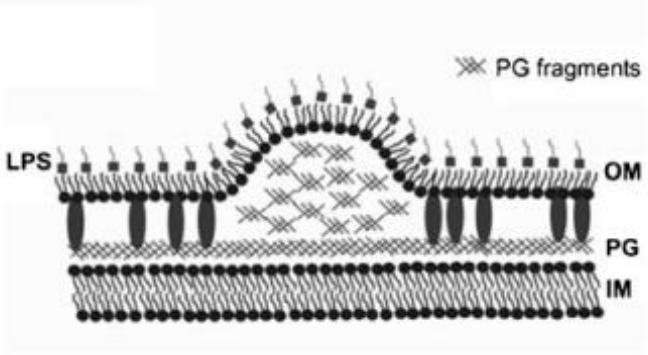

Figure 8. Model proposed for biogenesis of Gram negative bacterial OMV due to the action of β-lactam antibiotics. Accumulation of PG fragments in periplasm causes increased turgor pressure, thereby increasing blabbing of the OM (Lee & Choi 2008).

1.3.2.3. Efflux pumps

Bacterial efflux pumps (EPs) are proteins localized in the bacterial membrane that recognize noxious agents and extrude them from the cell. During excretory functions, EPs mobilize sources of energy to transport compounds against a concentration gradient (Amaral et al. 2014).

The principal efflux pump in Gram negative bacteria is the AcrAB-TolC-pump. It is composed by the AcrB protein attached to the plasma membrane, AcrA that flanks the AcrB transporter assisting in the movement of substrate, and the TolC protein which is a channel contiguous to AcrB. This efflux pump was shown to extrude a large variety of unrelated compounds, including hydrophilic drugs such as β-lactams (Masi & Pagès 2013). The expression of EPs is also controlled by different global regulators. For example, for the AcrAB-TolC-pump the transcriptional activators include MarA (involved in antibiotic resistance), SoxS (superoxide response regulator) and Rob (protein binding to the chromosomal origin of replication) (Kumar & Schweizer 2005). In *A. hydrophila* a similar AcrAB-TolC-pump was described and is called AheABC efflux pump (Hernould et al. 2008).

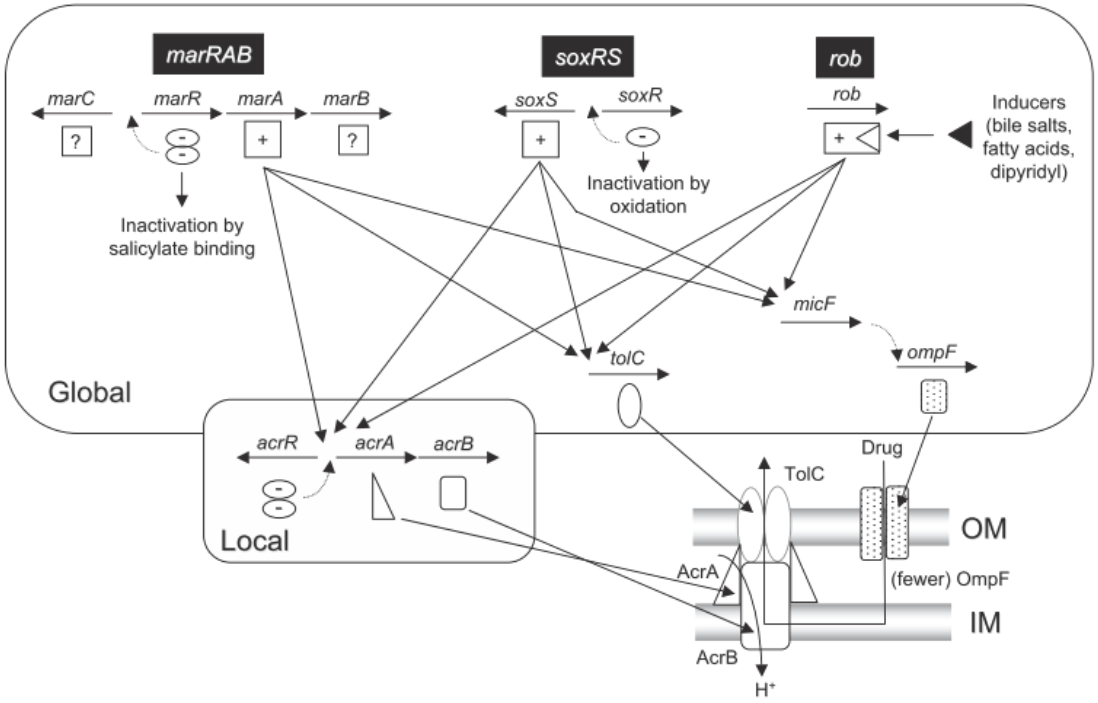

Figure 9. Global regulation of AcrAB-TolC pump. The *acrAB* operon is negatively regulated by a local repressor (*acrR*). *acrAB, tolC* and the antisense regulatory RNA *micF* are positively regulated by several activators (MarA, SoxS and Rob). The levels of MarA and SoxS are themselves regulated by the repressors MarR and SoxR, respectively. The activity of Rob is modulated by several metabolites. The *micF* transcript inhibits translation of the *ompF* porin mRNA, thus lowering expression of this outer membrane porin and reducing OM permeability for many drugs. At the same time, all three activators increase expression of AcrA, AcrB and TolC, leading to increased drug efflux (Kumar & Schweizer 2005).

1.3.3. Acquired resistance to β-lactam antibiotics

Resistance to β-lactams in *Aeromonas* spp. can be acquired by horizontal transfer of genetic elements and by mutation. DNA elements containing ARGs include plasmids, transposons and integrons. The spontaneous mutation frequency leading to resistant phenotype is dependent on the presence of DNA-damaging agents and/or the growth rate of bacteria.

1.3.3.1. Horizontal transfer of genetic elements

Aeromonas spp. have shown resistance to β-lactams through expression of relevant genes present in mobile genetic elements such as plasmids, transposons or integrons. In plasmids, different genes encoding β-lactamases were described: bla_{TEM-24} encoding an extended-spectrum β-lactamase (ESBL) (Fosse et al. 2004), bla_{FOX} encoding an AmpC-like β-lactamase (Maravić et al. 2013). In transposons bla_{KPC} encoding carbapenemase (Picão et al. 2013), bla_{SHV-12}, bla_{PER-1} encoding ESBLs (Girlich et al. 2011). In integrons, mainly in class 1, the genes bla_{OXA} (Henriques et al. 2006), bla_{CARB} (Igbinosa & Okoh 2012) and, bla_{VIM} (Libisch et al. 2008) were found, which encode an oxacillinase, a carbenicillinase and a carbapenemase, respectively. These genes present in *Aeromonas* spp. can confer resistance to β-lactam antibiotics and easily can spread (intra and interspecies level) representing serious risks to treatment of infection.

1.3.3.2. Antibiotic resistance by mutation

Stress is a disturbance of the normal functioning of a biological system that is potentiated by several factors, the amplitude and persistence of which being causes of reduced growth rate or increased mortality. Different stresses as sub inhibitory concentrations of antibiotics can increase the mutation rates in bacteria. This can be an adaptive strategy that could potentiate the survival (Blázquez 2003).

β-lactams inhibit the cell wall synthesis inducing the SOS system. The SOS response involve several metabolic changes that consequently modify the activity of the error-prone DNA polymerase and lead to an increase of the mutations, finally contributing to acquired resistance (Blázquez 2003; Tenaillon et al. 2004).

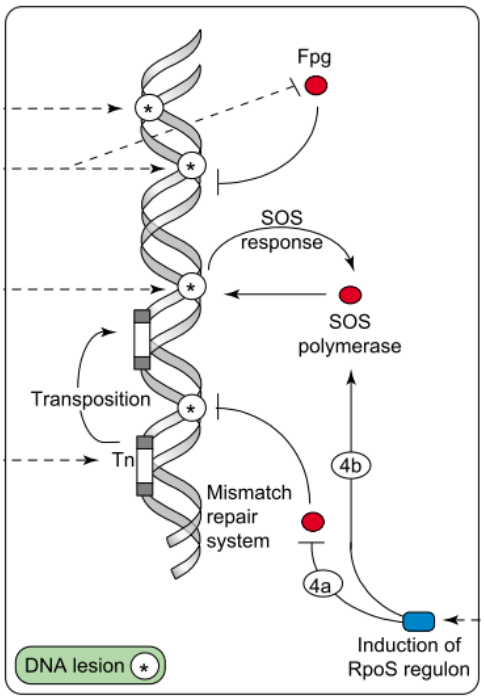

Figure 10. Molecular mechanisms associated with stress-induced mutagenesis (Tenaillon et al. 2004)

1.3.3.2.1. Oxidative stress

Reactive oxygen species (ROS) are generated by normal cellular metabolism in aerobic conditions. Bacterial cells produce superoxide dismutase and catalases/peroxidases to enzymatically eliminate ROS (Dwyer et al. 2009). The presence of hydroxyl radicals can damage the DNA through strand breaks, deamination and depurination. In addition, oxidation also generates 2'-deoxy-7,8-dihydro-8-oxoguoanosine (8-oxo-dG) which pairs preferentially with adenine leading to a transversion event (Chopra et al. 2003).

The antibiotic exposure can also generate ROS, increasing the oxidative stress in bacterial cell and promoting cell death (Kohanski et al. 2007). However, the bacterial cell normally adapts producing a SOS response that mitigates the genotoxic effects of hydroxyl radicals. This SOS response leads to an increase in protecting proteins know as molecular chaperones (Calloni et al. 2012) and in NADH production by the tricarboxylic acid cycle, decreasing superoxide generation (Kohanski et al. 2007). This response against oxidative stress was also observed in *A. hydrophila* (Landre et al. 2000).

A balance between various processes mentioned before can allow bacteria to survive and also adapt to stressful conditions mainly due to the control of oxidative stress.

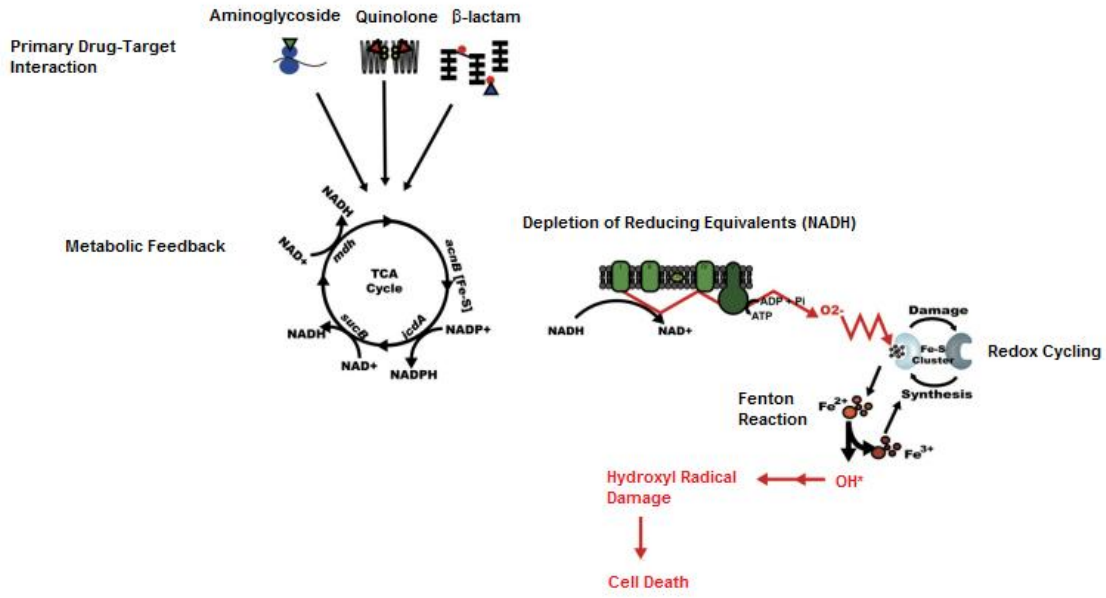

Figure 11. Model for common mechanism of killing by antibiotics (Kohanski et al. 2007)

1.3.3.2.2. Bacterial persistence

This type of response involves toxin-antitoxin (TA) modules which contribute to the persisters formation (Wu et al. 2012), which are dormant variants of normal cells. TA modules consist of two genes in one operon encoding a stable toxin that disrupts an essential cellular process and a labile antitoxin that neutralize toxicity by binding to the protein or to the mRNA of the toxin (Hong et al. 2012). In the context of an SOS stress response there is an overexpression of toxin TisB and this protein allow persister formation (Lewis 2010). The persister formation consists in the induction of the dormant state that allows bacteria survival. This happens because the cell is able to adapt quickly by switching on/off genes linked to general stress responses (Gefen & Balaban 2009) .

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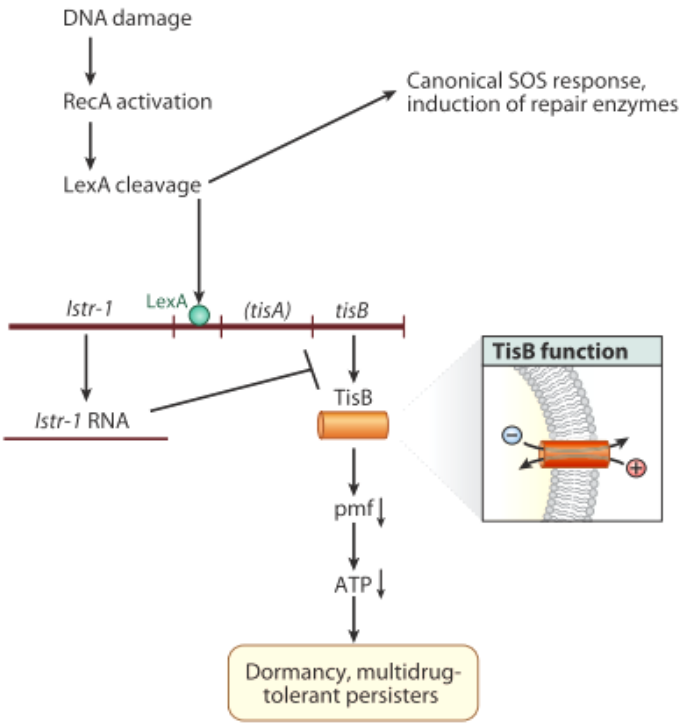

Figure 12. SOS response induce persister formation (TisB-dependent). The antibiotic exposure causes DNA damage, this activates the RecA protein, which in turns activates the LexA repressor. The canonical SOS response is induced, and repair enzymes that contain *lex* boxes in their promoter regions are transcribed. The Lex repressor also controls the expression of the TisB toxin, a small cationic membrane-acting agent. Decrease in the proton motive force (pmf) and ATP shuts down target functions, including DNA topoisomerase and gyrase, and a dormant persister is formed (Lewis 2010).

1.3.4. Adaptive resistance to β-lactam antibiotics

Adaptive resistance is inducible and depends on the presence of either an antibiotic or another environmental stimulus. This mechanism comprises all the adaptations that occur in intrinsic and acquired mechanisms during the development of resistance. In *Aeromonas* the major adaptive mechanism is the induction of chromosomally-encoded β-lactamases by pre-exposure to β-lactam antibiotics. Several studies have demonstrated the occurrence of this adaptive response in *Aeromonas* spp. (Alksne & Rasmussen 1997; Avison et al. 2004; Tayler et al. 2010). Another adaptive mechanism resulting from exposure to antibiotics is the changes in membrane permeability observed in *A. salmonicida*

when challenged by low concentrations of β-lactam antibiotics (Wood et al. 1986). This adaptation process leads to a lower exoprotease activity (Wood et al. 1986). The overexpression of EPs like the AheABC efflux pump described in *A. hydrophila* (Hernould et al. 2008), or the aggregation/biofilm formation observed in aquatic bacterial communities that include *Aeromonas* spp. (Corno et al. 2014), are examples of the result of the adaptive response.

All these mechanisms include involvement of complex physiological responses important to explain this adaptive resistance but poorly described in genus *Aeromonas.*

1.4. PROTEOMIC ANALYSIS OF BACTERIAL RESISTANCE

Infectious diseases have been estimated to be the second leading cause of death in the world, mainly due to an increase of resistant strains (Radhouani et al. 2012). Bacteria can acquire genetic determinants, consequently expressing new characteristics mainly when subjected to stressful conditions, like sub inhibitory concentrations of antibiotics. The stress response can lead to a successful cascade of adaptation; however this process involves coordinated expression of genes that alter different cellular processes and act collectively to improve the bacterial tolerance (Radhouani et al. 2012).

Identify regulators and regulatory networks involved in these processes are essential to control bacterial behaviour. Hence, proteomic approaches are powerful tools for this endeavour (Renzone et al. 2005).

In general, proteomics allow the description of protein expression profiles, providing a large-scale characterization of all identified proteins in a specific physiological state. Proteomics also informs about post-translational alterations in proteins, allows to compare protein expression levels in two or more physiological states, gives information about protein-protein interactions and, subcellular protein localization, showing their biological role (Radhouani et al. 2012).

1.4.1. Comparative proteomics

In comparative proteomic studies, proteins from different biological states are quantitatively compared to obtain a thorough understanding of the biological processes affecting their expression or/and the processes in which they are involved. There are several methodologies that have been developed to achieve this goal (Radhouani et al. 2012).

Two-dimensional electrophoresis (2-DE) is the most widely used separation methodology and is used with or without previous sample enrichment using other techniques. Initially, proteins are separated and then quantified following suitable chemical staining comparing different physiological states or

treatments. Proteins with differential expression are finally identified by mass spectrometry procedures (MS). This type of technique is included in gel-based proteomic techniques (Chambers et al. 2000).

This kind of method presents many advantages mainly related to high resolution power and good visualization of the results. However it also has some limitations, namely reproducibility, poor representation of low abundant proteins, poor representation of the highly acidic/basic proteins or proteins with extreme size or hydrophobicity and difficulties in automation. Therefore a technique that reduces some of these errors was developed, called 2D-DIGE (2D- difference gel electrophoresis). This technique uses fluorescent dyes which have an NHS-ester reactive group that covalently attaches to the ε-amino group of protein lysines via an amide linkage. This technique allows a better detection of low abundant proteins and permits to run and compare simultaneously two biological samples on the same gel using different labelling for each sample (Cy3 or Cy5). An internal standard can be used (mixture of all samples in the experiment labelled with Cy2) thus reducing technical variability and increasing statistical power (Abdallah et al. 2012; Renzone et al. 2005).

At the present, other techniques can be used in proteomic approaches, namely those that avoid protein separation in gel (non-gel based proteomics). In this type of techniques a complex mixture of proteins is digested and the resulting peptide mixture is separated by various chromatographic techniques and then analyzed by the mass spectrometer to obtain sequence information (LC MS-MS). As separation and identification are performed at peptide level, this approach allows to access groups of proteins that may have not been identified by 2-DE techniques. However, comparative expression studies need the introduction of stable isotope labelling reagents (Renzone et al. 2005; Abdallah et al. 2012; Roe & Griffin 2006).

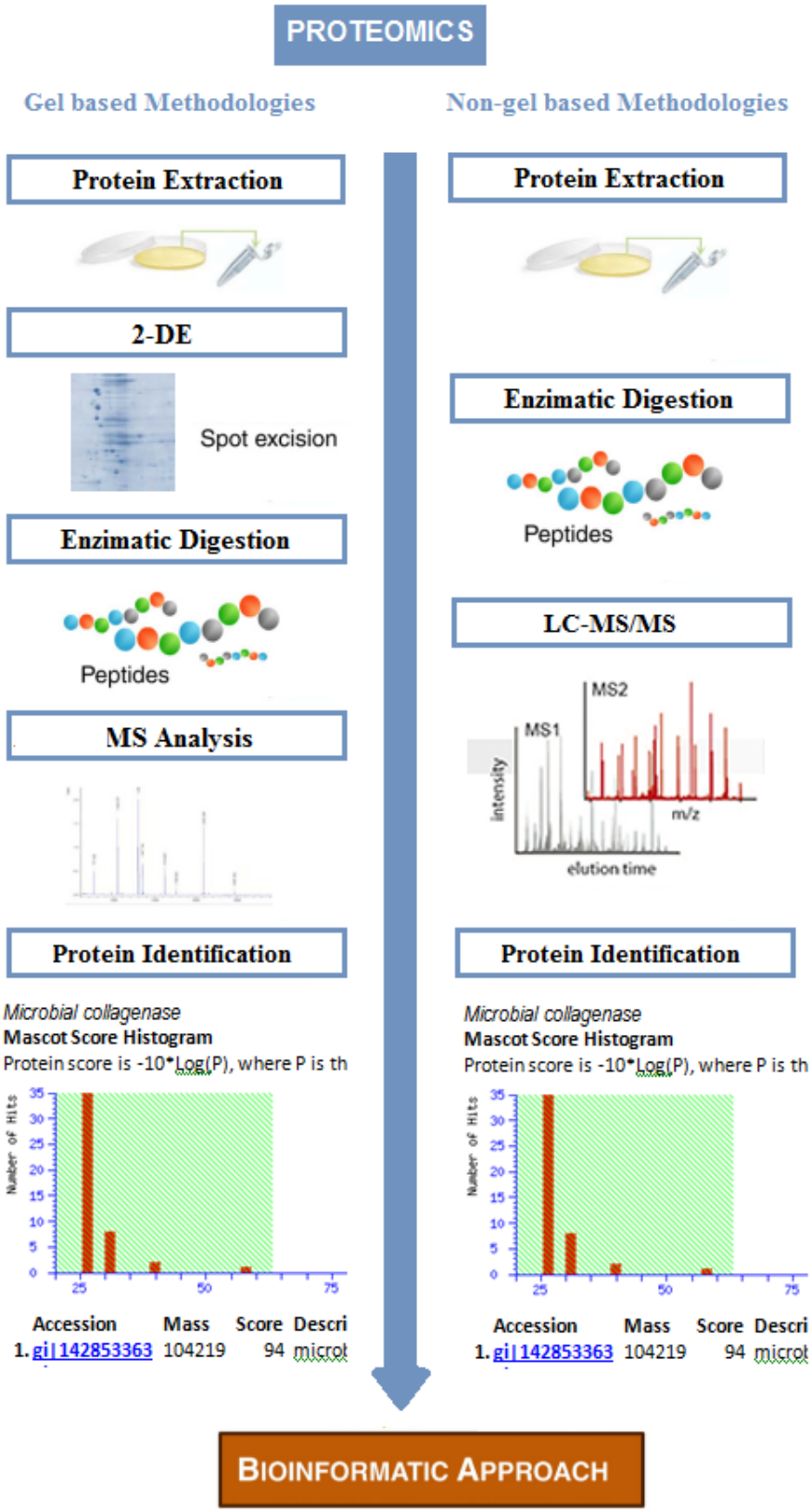

Figure 13. Proteomic workflow representing gel-based and non-gel based techniques to protein identification.

1.5. SCOPE OF THIS THESIS

The aim of this study is to characterize the response of *A. salmonicida* to an ampicillin challenge. This study represents an attempt to fill some pieces of the puzzle that constitutes the β-lactam "resistome" in *Aeromonas* and to get a systematic understanding of its functions.

To accomplish this, specific purposes were outlined:

- To produce an ampicillin resistant strain using *A. salmonicida* CECT894^T as the source strain.

- To characterize the phenotypic behaviour of *A. salmonicida* strains when challenged by subinhibitory concentration of ampicillin:
    - namely in what concerns their growth rates, levels of β-lactamase production and expression of virulence factors (exotoxins such as haemolysins and proteases, cytotoxins and biofilm formation).

- To compare the intra- and extracellular protein profiles of both strains by:
    - identifying proteins differentially expressed;
    - establishing their biological role;
    - and, predicting protein-protein interactions.

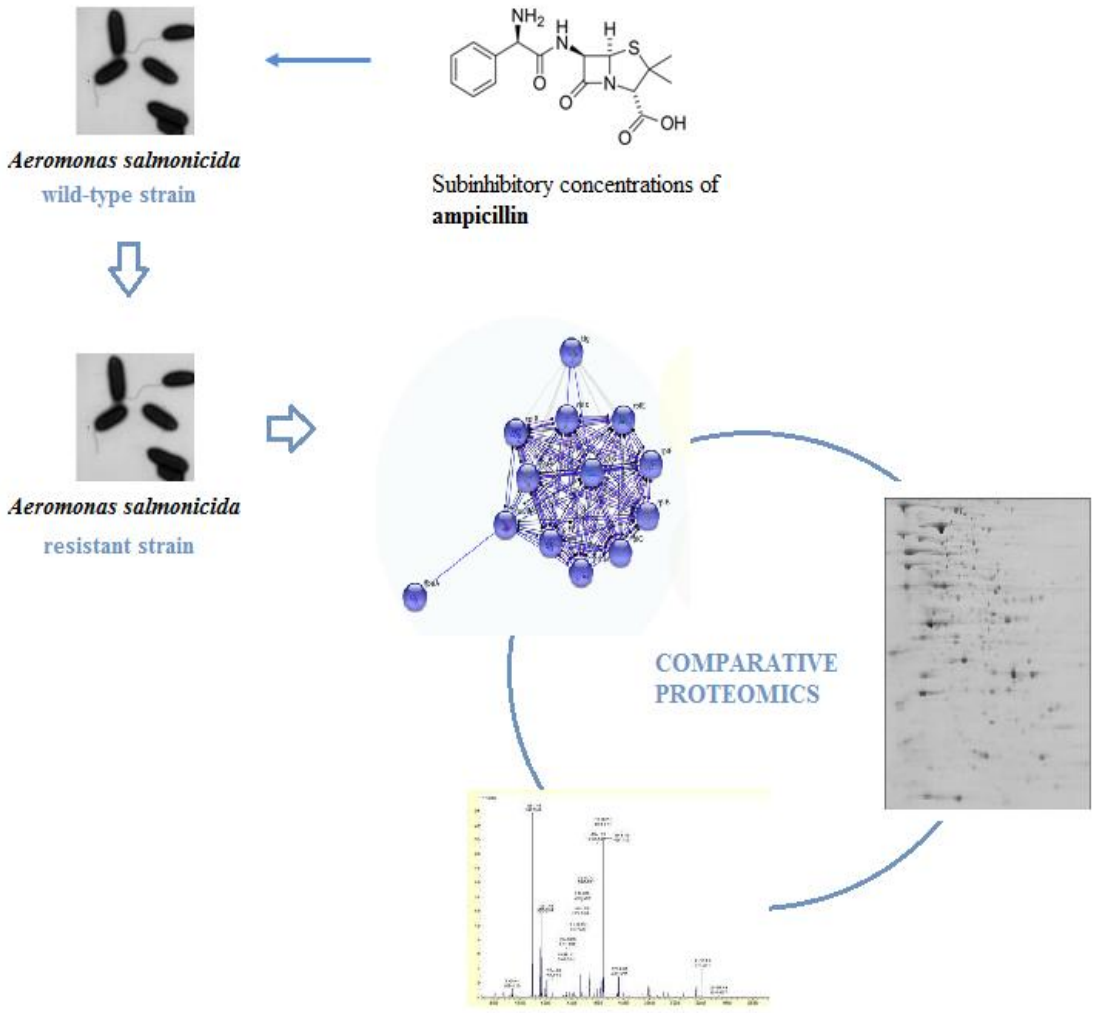

Figure aim 1. Workflow of this phD thesis: unravelling some of the adaptation mechanisms of *A. salmonicida* to survive and resist an ampicillin challenge.

CHAPTER II

STUDY I

2. PHENOTYPIC BEHAVIOR OF *AEROMONAS SALMONICIDA* STRAINS WHEN CHALLENGED BY AMPICILLIN

2.1. ABSTRACT

Exposure to subinhibitory concentrations of antibiotics, although not causing bacterial death or inhibiting its growth, induces phenotypic modifications. In this study, we induced resistance experimentally in *A. salmonicida* CECT894^{T} (As^{WT}), creating a derivate ampicillin resistant strain (As^{R}). Genetic relatedness between strains was assessed by PCR-based typing techniques. Susceptibility to other antibiotics was assessed for both strains using a disc diffusion method. We evaluated the influence of sub-MIC of ampicillin on parameters like expression of β-lactamases, growth rate, expression of virulence factors (haemolysins, proteases, production of cytotoxins) and biofilm formation. Antimicrobial susceptibility was determined by the E-test method. The effects of sub-MICs of ampicillin on β-lactamases activities were determined by kinetics reading against antibiotics. Growth rates were determined by spectrophotometric measurements. Production of hemolysins and proteases was evaluated by culturing onto agar plates with adequate medium. Cytotoxicity was evaluated by a resazurin assay against vero cells line. Biofilm formation was measured by the microtitre plate assay using the crystal violet method. Strains were genetically identical but the As^{R} strain presented a resistant phenotype not only to ampicillin but also to other β-lactams. The results obtained showed that subinhibitory concentrations of ampicillin reduce the growth rate, increase the expression of inducible β-lactamases and increase the ability to form biofilm, important processes that are related to stress response. However, changes in production of hemolysins and proteases and the increase in cytotoxicity seem not to be directly related to the exposure to the antibiotic but instead are a consequence of the initial phenotype of susceptibility to antibiotics.

Key words: subinhibitory concentrations, *Aeromonas* spp., β-lactamases expression, virulence factors

2.2. INTRODUCTION

The genus *Aeromonas* is widely distributed in the environment. Its members are found on natural soil, food, animals, but most commonly arise in diverse aquatic environments such as wastewater, rivers, lakes, estuaries, aquacultures or urban drinking water (Piotrowska and Popowska 2014; Parker and Shaw 2011). Some species of this genus emerged as important human pathogens, able to cause intestinal infections such as gastroenteritis, bacteremia, skin and soft tissues infections or even sepsis with lethal course in immunocompetent and immunocompromised individuals (Carvalho et al. 2012; Ghenghesh et al. 2008). In animals, they also stand out as able to cause diseases in amphibians, reptiles and fish, with higher impact in salmon aquaculture's due to high economic losses caused by furunculosis, septicemia, ulcerative and hemorrhagic diseases (Dallaire-Dufresne et al. 2014).

The *Aeromonas* spp. infections are particularly difficult to treat due to multifactorial virulence depending mainly on the intrinsic resistance to a broad spectrum of antimicrobials agents and its repertoire of virulence factors (Carvalho et al. 2012). The major mechanism of antibiotic resistance recognized in *Aeromonas* species consists of chromosomally mediated, inducible β-lactamases. *Aeromonas* species possess at least three inducible chromosomally encoded β-lactamases: the class B CphA, also known as ImiS, a carbapenemase, the class C cephalosporinase Cep; and a class D penicillinase Amp (Chen et al. 2012). The expression of the genes encoding these enzymes is coordinated by a common regulatory pathway (Niumsup et al. 2003, Alksne and Rasmussen 1997). The paradigm for regulation of β-lactamases production in Gram negative bacteria is the AmpC/AmpR system. However, in *Aeromonas* spp. the coordinated expression of multiple β-lactamases involve a two component system (TCS) and response regulators (RR) phosphorylation dependent, the *blrAB* system (Avison et al. 2004). Although the expression of inducible β-lactamases can be regulated differently, a challenge by antibiotic is always needed for mechanism activation. A monomer of PG found in high levels in cytoplasm, M5 (monomer disaccharide-pentapeptides), activates BlrB (a sensor kinase) and this, in turn activates the

transcription factor BlrA (a phosphorylation dependent response regulator) that regulates the expression of β-lactamases genes (Tayler et al. 2010).

The development of infection by *Aeromonas* spp. is dependent on the expression of virulence factors such as exotoxins (hemolysins, cytotoxins, enterotoxins, proteases, leukocidin, phospholipases and proteins like siderophores) and endotoxins (surface associated factors like adhesins) (Chopra & Houston 1999). The expression of many of these virulence determinants is associated with high cell densities and are therefore putatively controlled by QS (Swift et al. 1997; Jangid et al. 2007).

QS is a mechanism that controls gene expression in parallel with the expansion of the bacterial population and is mediated by signal molecules such as the N-acyl-homoserine lactones (AHLs) (Swift et al. 1997). Two AHLs are produced by *Aeromonas* spp., namely N-3-butanoyl-DL-homoserine lactone (C4-HSL) and N-3-hexanoyl-DL-homoserine lactone (C6-HSL), of which C4-HSL is predominant. These molecules are synthesized by the LuxI protein family and activate proteins of the LuxR family when they reach a certain concentration (autoinducer I). The LuxR protein family is able to regulate the expression of *luxI* gene as well as the expression of many genes involved in biofilm formation, motility and virulence (Khajanchi et al. 2009). Other QS systems were described in *Aeromonas* spp. such as LuxS (autoinducer-2) and the QseBC signalling system (autoinducer-3) (Kozlova et al. 2011; Khajanchi et al. 2012). The regulation of all QS systems identified in *Aeromonas* spp. are dependent of cyclic diguanosine monophosphate (c-di-GMP), a bacterial intracellular second messenger responsible to produce a signaling pathway cascade that leads to transcription of many genes (Kozlova et al. 2011; Khajanchi et al. 2012).

In *Aeromonas* spp., the two-component system is very important for signal transduction facilitating adaptive responses to several environmental stimuli. Normally, the stimulus promote phosphorylation events that are sensed by response regulators, which in turn respond by conformational changes that subsequently exert regulation of transcription of various genes (Tayler et al. 2010; Khajanchi et al. 2012).

In this study, we exposed continuously the *A. salmonicida* CECT894^{T} strain (As^{WT}) to subinhibitory concentrations of ampicillin (stimulus) and by doing this we obtained a resistant strain (As^{R}). After that, we evaluated the effect of exposure to antibiotic for both strains particularly in what concerns changes on activity of β-lactamases, on production of virulence factors (i.e. hemolysins and proteases), and in complex phenotypes like cytotoxicity and biofilm formation.

2.3. MATERIAL AND METHODS

2.3.1. In vitro selection of resistant *A. salmonicida* strain by exposure to subinhibitory concentration of ampicillin

A. salmonicida CECT894^T was used as wild-type strain (As^{WT}). A resistant strain was obtained by serially subculturing the wild type strain in Muller Hinton Agar (MHA) (Merck KGaA, Germany) containing a linear gradient of ampicillin. Ampicillin (AppliChem GmbH, Germany) solutions were freshly prepared in 0.1M of phosphate buffer, pH 8. MHA plates with gradient of antibiotic were prepared as previously described by Szybalski and Bryson (1952). All cultures were incubated at 30ºC and the cultures in LB were incubated with shaking at 180rpm.

The As^{WT} grown overnight in Luria Broth (LB), and then diluted to an optical density (OD) at 550nm of 1.2. After, 100 µL (containing approximately 10^9 CFU) were homogeneously spread onto MHA-ampicillin gradient plates. For the first gradient plate the maximal concentration of ampicillin was 2x the MIC (minimum inhibitory concentration) for wild type strain (2x0.5µgmL^{-1}). Following 20h of incubation, the leading edge of growth was sampled with a loop and subcultured into fresh LB without antibiotic. The new culture was diluted and plated as described for the initial passage. The antibiotic concentration in gradient plate was increased twofold once growth was observed at approximately half of the plate distance.

2.3.2. *In vitro* susceptibility test

The susceptibility to antibiotics of As^{WT} and the resistant strain (As^R), was determined by the Kirby-Bauer disc diffusion method, as recommended by the CLSI (2010) guidelines. The following antibiotics were tested: amikacin (AMK; 30 µg), cefotaxime (CTX; 30 µg), aztreonam (ATM; 30 µg), imipenem (IPM; 10 µg), gentamicin (GEN; 10 µg), kanamycin (KAN; 30 µg), tetracycline (TET; 30 µg), chloramphenicol (CHL; 30 µg), trimethoprim/sulfamethoxazole (SxT; 25 µg) and ciprofloxacin (CIP; 5 µg) (Oxoid, UK). The MIC to ampicillin was determined by E-test method (Biomérieux SA, France), according to the CLSI (2010).

2.3.3. Growth rates

As^{WT} and As^{R} cultures were grown in LB and LB with subinhibitory concentrations of ampicillin (½ MIC of As^{WT}). This experiment was made in two independent assays for each condition. The OD_{550nm} was adjusted to 0.05 corresponding approximately to 10^6 cells mL^{-1}. These suspensions were then incubated at 30ºC, during 24h with agitation at 180rpm. Throughout the growth aliquots were removed for subsequent reading of OD at 550nm on a spectrophotometer UV-VIS (mini-1240, Shimadzu).

2.3.4. Genotypic profiles of As^{WT} and As^{R} strains

The genetic relatedeness of As^{WT} and As^{R} strains was inspected by rep-PCR analysis. The primers used in this study were REP1R (5′-IIIICGICGICATCIGGC-3′) and REP2I (5′-NCGICTTATCIGGCCTAC-3′) for REP-PCR; ERIC1 (5′-AAGTAAGTGACTGGGGTGAGC-3′) and ERIC2 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) for ERIC-PCR; and BOX A1R (5′-CTACGGCAAGGCGACGCTGAC-3′) for BOX-PCR. The reaction mixtures (25µL) used for the three sets of primers consisted of 6.25 µL NZYTaq 2× Green Master Mix (2.5mM $MgCl_2$; 200µM dNTPs; 0.2U/µL DNA polymerase) (NZYtech, Portugal), 15.75µL of ultrapure water and as for the primers, 2µL of a 10µM BOX A1R primer, and 1µL of 10µM solutions of ERIC and REP primers. PCR reactions were performed using a MyCycler Thermal Cycler (Bio-Rad, USA). The amplification conditions consisted of an initial denaturation step (95 °C for 7 min), followed by 30 amplification cycles consisting of denaturation (94 °C for 1 min), annealing (variable temperature for 1 min) and extension (65 °C for 8 min), and a final extension step (65 °C for 16 min). The annealing temperature was 40 °C for REP-PCR, 52 °C for ERIC-PCR and 53 °C for BOX-PCR. A negative control (no DNA) was also always included. The reaction products were separated by electrophoresis on a 1.5% (w/v) agarose gel together with a 1-kb molecular weight ladder (GeneRuler DNA LadderMix, MBI Fermentas, Lithuania). All gels were run in 1×TAE buffer at 80V for 80min, stained in 0.5µgmL^{-1} ethidium bromide solution and images were acquired with the Gel DocMega camera system 5.01 (Biosystematica, UK).

2.3.5. Phenotypic profiles of As^{WT} and As^{R} strains challenged by ampicillin

2.3.5.1. Bacterial strains and culture conditions

The As^{WT} and As^{R} strains were cultured in LB (at least six biological replicates of each). Half replicates of each type were grown in the absence of ampicillin, and the remaining biological replicates were grown in the presence of ampicillin at a final concentration corresponding to ½ MIC (As^{WT}, 0.25µgmL^{-1} and As^{R}, 64 µgmL^{-1}). All cultures were incubated at 30ºC with shaking at 180rpm until an OD_{550nm} of 0.9 was reached.

2.3.5.2. β-Lactamase assay

Bacterial strains were grown as described in a previous mentioned. The cell pellets were collected by centrifugation at 13000xg for 10 min at 4ºC. After being washed twice in ice cold extraction buffer (10mM phosphate buffered saline pH 7.4), they were dissolved in 1mL of the same buffer containing (EDTA-free) protease inhibitor mixture (GE Healthcare Europe GmbH, Germany) and sonicated three times with 15s pulses and 30s intervening periods. This process was made in ice to minimize β-lactamase damage. The protein solution obtained was subsequently centrifuged at 1500xg for 20 min at 4ºC and the supernatant was retained as a crude enzyme preparation (Ko et al. 1998).

Protein concentrations for these bacterial extracts were determined in triplicate using the *DC*™ Protein Assay Kit (Bio-Rad laboratories**,** USA). Appropriate dilutions of protein extracts were employed for the enzymatic reaction. AmpS, CepS and ImiS activities were measured using different antibiotics (at 100µM concentration), namely ampicillin, cefotaxime and imipenem. The kinetics measurements were performed at 25ºC in 10mM phosphate buffered saline pH 7.4, using a spectrophotometer UV/VIS Lambda 14P (Perkin Elmer) with readings every 10s during 5min, operating at the wavelength of maximum absorbance for the antibiotic (for ampicillin, 233nm; for cefotaxime, 265nm; for imipenem, 295nm). The rate of hydrolysis was expressed as substrate hydrolyzed per minute per milligram of protein (Ko et al. 1998). This experiment

was performed with six biological replicates for each condition and three replicates for each technique.

2.3.5.3. Biofilm formation assay

Cultures obtained as mentioned before, were adjusted to an OD_{550nm} of 0.05 using fresh LB and LB with ampicillin, respectively. Then, 1mL of these suspensions was inoculated in a 12 wells microplate and incubated at 30ºC during 24h. Planktonic bacteria were transferred to a new 96 wells microtiter plate and the OD at 600nm was read. Non-adherent cells were removed by washing the wells twice with 10mM phosphate buffered saline pH 7.4. Adherent cells constituting a biofilm were quantified according to procedures previously described by Merritt et al. (2005). All reagents were used at the same concentrations and the volumes were adjusted for final resuspension of 1 mL. This experiment was performed with three biological replicates and three replicates for each technique. Biofilm formation results were normalized using the ratio of adherent cells at OD_{600nm} / planktonic cells at OD_{550nm}.

2.3.5.4. Resazurin based cytotoxicity assay

Cultures were centrifuged at 8.000xg for 10min at 4ºC and the supernatants were filtered through a sterile 0.2µm pore size syringe membrane filter. The extracellular products (ECP) were collected in sterile tubes and stored at -80ºC until use for *in vitro* cytotoxicity evaluation. The cytotoxicity assay was performed according to Cruz et al. (2013), modifying the dilutions of ECP for 1:1; 1:4; 1:8 (v:v). Each sample was tested in three independent experiments performed in triplicate. Percentage of viable cells was calculated by [($OD_{570/600nm}$ cell treated - $OD_{570/600nm}$ negative control) / ($OD_{570/600nm}$ positive control - $OD_{570/600nm}$ cell treated)]*100. The morphology of cells on Vero cell monolayers was evaluated by observations using inverted light microscopy. Images were acquired using a CKX41 Olympus inverse microscope with a digital colour camera Olympus CAM-SC30 and a 20x objective.

2.3.5.5. Hemolytic activity

Hemolytic activity was investigated by culturing the strains onto Columbia gelose with 5% sheep blood (Biomerieux, France). The plates were incubated at 30ºC for 24h *A. hydrophila ATCC 7966* was used as a positive control. Haemolytic positive isolates were identified by the presence of clear (β-hemolysis) or diffuse (α-hemolysis) halos around the colonies (Cruz et al. 2013).

2.3.5.6. Proteolytic activity

Proteolytic activity was investigated by culturing the strains in TSA supplemented with 1% skim milk (w/v) (Biomerieux, France). The plates were incubated at 30ºC for 24h. *A. hydrophila* ATCC 7966 was used as a positive control. The presence of extracellular proteases was revealed by the formation of clear halos around the colonies (Cruz et al. 2013). The halos were measured, and the results were expressed in % with data from the control strain (As^{WT}) considered as 100%.

2.3.6. Data Analysis

Statistical analyses were performed using GraphPad Prism v.5 software (GraphPad Software Inc.). For data of protease activity, β-lactamases activities and biofilm formation assays a one-way ANOVA through Bonferroni's Multiple Comparison Test was used. For cytotoxicity assays a two-way ANOVA through Bonferroni post tests was used. Differences were considered statistically significant when $\rho_{value} < 0.05$.

2.4. RESULTS AND DISCUSSION

The *A. salmonicida* strain resistant to ampicillin (As^R) was obtained from the wild type strain (As^{WT}) after 16 serial passages on plates containing an antibiotic gradient, according to the methods described. Incubation of *A. salmonicida* CECT894^T with subinhibitory concentrations of ampicillin produced a 256-fold MIC increment (from 0.5 to 128µgmL^{-1}) (Figure 1).

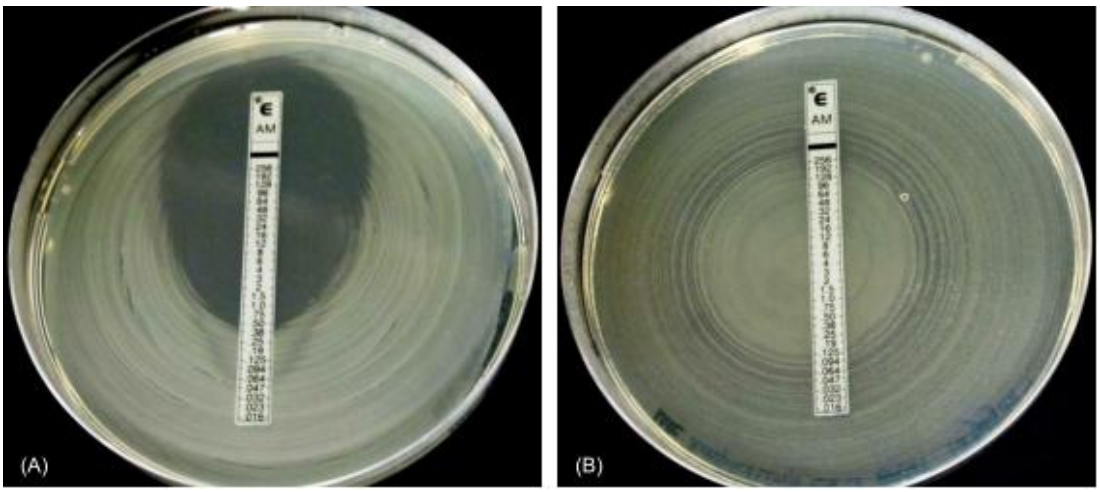

Figure 1. Antimicrobial susceptibility test, by diffusion method: E-test to ampicillin. (A) Minimum inhibitory concentration (MIC) of *A. salmonicida* CECT894^T, wild-type strain (As^{WT}), 0.5µgmL^{-1}. (B) MIC of *A. salmonicida* derivate resistant strain (As^R), 128µgmL^{-1}.

Minimum inhibitory concentrations (MICs) of *A. salmonicida* CECT894^T, the wild-type strain, As^{WT} (MIC 0.5µgmL^{-1}), and of *A. salmonicida* derivate resistant strain, As^R (MIC 128µgmL^{-1}) were determined by E-test, as shown in Fig. 1. Antibiotic susceptibility testing by disc diffusion method allowed verifying that As^R is resistant to cefotaxime and impinem whereas As^{WT} is sensitive. For the remaining antibiotics, no differences in the antimicrobial profiles were observed. These results corroborate the previous observation by Walsh and co-workers, supporting a coordinate regulation of two or three inducible β-lactamases in *Aeromonas* (Walsh et al. 1997).

The fingerprint analysis by rep-PCR showed no differences in the genotype profiles of As^{WT} and As^{R}. These results demonstrate that these strains are genetically similar and that resistance acquisition to β-lactam antibiotic is most probably due to an adaptive response involving changes in gene expression. (Figure 2).

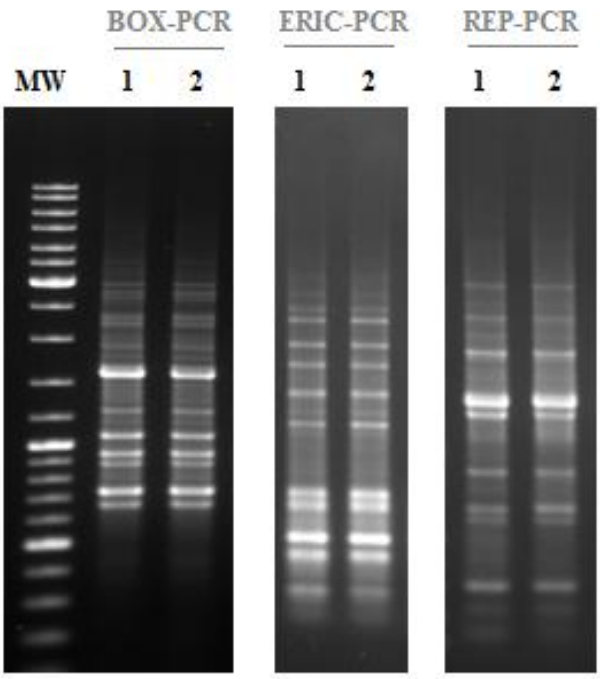

Figure 2. Genotypic profiles of As^{WT} (1) and As^{R} strains (2) by *rep*-PCR (BOX-, ERIC- and REP- PCR).

2.4.1. Effect of subinhibitory concentrations of ampicillin on growth rate

In a bacterial population, even if it is a pure culture, cells coexist that are rapidly inhibited or killed by antibiotics and cells that suffer those processes very slowly. The cells that respond slowly are named “persisters” and are in fact tolerant cells under stress conditions. These cells display phenotypic variants of the wild type strain and represent a small subpopulation (~1%) that spontaneously enter a dormant, nondividing state (Gefen and Balaban 2009). This nondividing state permits rapid adaptation for instance by switching on/off genes linked to general stress responses. One study evaluating persister cells of *Escherichia coli* under antibiotic pressure, demonstrated overexpression of proteins that can block cellular functions: several TA (toxin-antitoxin) module proteins, RMF (inhibitor of

translation) and UmuDC (inhibitor of replication) (Keren et al. 2004). This process may help to explain the observed changes in the growth profile of the *Aeromonas* strains that were submitted to antibiotic stress in present study (Figure 3).

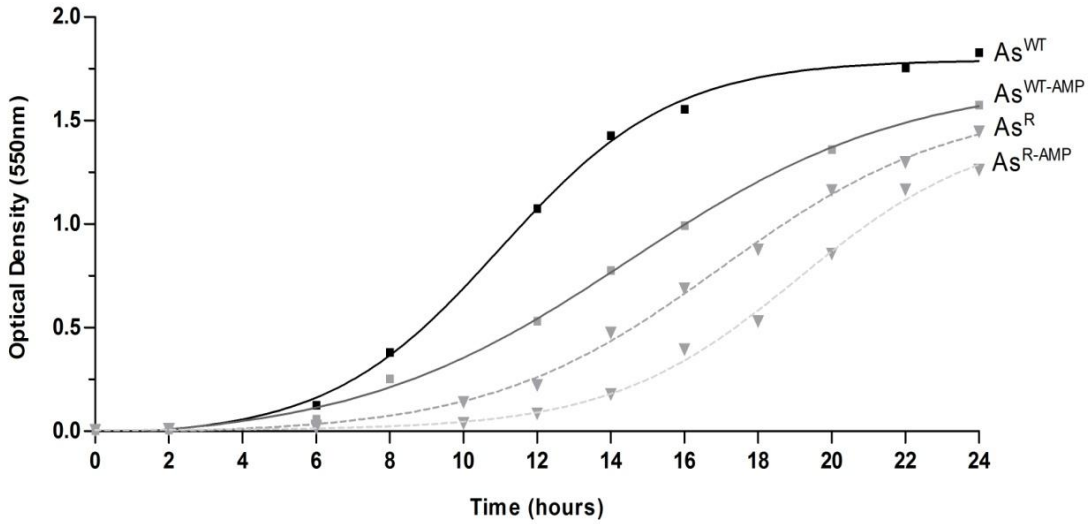

Figure 3. Growth rates of *A. salmonicida* CECT894^T, wild-type strain (As^{WT}), *A. salmonicida* CECT894^T, wild-type strain challenged by ampicillin (As^{WT-AMP}), *A. salmonicida* resistant strain (As^R) and *A. salmonicida* derivate resistant strain challenged by ampicillin (As^{R-AMP}).

Furthermore, the β-lactam antibiotics such as penicillins and cephalosporins act by inhibiting the cell wall biosynthesis. The cell wall contains PG, which is responsible for maintaining cell integrity by sustaining internal osmotic pressure and helps to keep the regular bacterial shape. Its biosynthesis involves multi stage enzymatic activities, including the transpeptidases that are the targets of β-lactams antibiotics, the so-called PBP's (Zeng and Lin 2013; Comber et al. 1977). The PBP's are known to be essential for cell elongation and septation, in such a way that its inactivation influences negatively the cell growth and subsequent division (Laubacher and Ades 2008).

The lower growth rates observed in the resistant strain even in the absence of antibiotic (As^R) can be a consequence of physiological changes that occur in bacterial cells and contribute to lower the phenotype of sensitivity to the

antibiotic. For example, resistant cells are able to change membrane permeability, consequently lowering nutrient diffusion and consuming less energy, thus displaying altered metabolic pathways and modifications in growth rate. Associations between energy production, metabolic changes, persistence phenotype and antibiotic resistance were observed in *E.* coli and *Pseudomonas aeruginosa* (Ma et al. 2010; Linares et al. 2010).

2.4.2. Inducible β-lactamases in response to a challenge of ampicillin

Chromosomally mediated inducible β-lactamases were recognized as the major mechanism of antibiotic resistance in *Aeromonas* species. *A. salmonicida* possesses three inducible β-lactamases (AmpS – penicillinase; CepS – cephalosporinase; ImiS – carbapenemase) and their encoding genes are coordinately expressed due to a common regulatory pathway (Walsh et al. 1997). The regulation mechanism of these β-lactamase genes is called *blrAB* system (Niumsup et al. 2003). BlrB molecule is a signal sensor and is responsible for BlrA activation. This last molecule is a transcription factor of β-lactamase genes. However, the expression of different β-lactamases genes can still be different depending on the number of short DNA sequences called blr-tags, located upstream of the genes, as was demonstrated in *A. hydrophila* T429125 (Avison et al. 2004). This mechanism can explain our results for differential β-lactamase activity when we tested the responses of the same strains to different antibiotics (Figure 4).

The *blrAB* system, in a similar way to the AmpG-AmpR-AmpC system (which is a regulation mechanism of β-lactamase production in other Gram negative bacteria) is activated upon β-lactam challenge (Zeng and Lin 2013, Tayler et al. 2010). It was shown for *Aeromonas* spp. 163a, that the *blrAB* mechanism of induction is dependent on the cytoplasmic concentration of M5 (disaccharide-pentapeptide monomer). This monomer, results from inactivation of PG biosynthesis when β-lactam antibiotic binds to PBPs. The increase of cytoplasmic concentration of this component is detected by the sensor domain BlrB and this develops a cascade of activations culminating in β-lactamase expression (Tayler et al. 2010). This explains the higher level of β-lactamase

activity in *Aeromonas* strains that were previously grown in the presence of ampicillin. And still it is clear, why $As^{R\text{-}AMP}$ showed higher β-lactamase activity: firstly, because this strain uses the mechanism mentioned for increase the beta-lactamases expression when grown in the presence of ampicillin and then because the initial phenotype proportionate a higher basal levels of these enzymes.

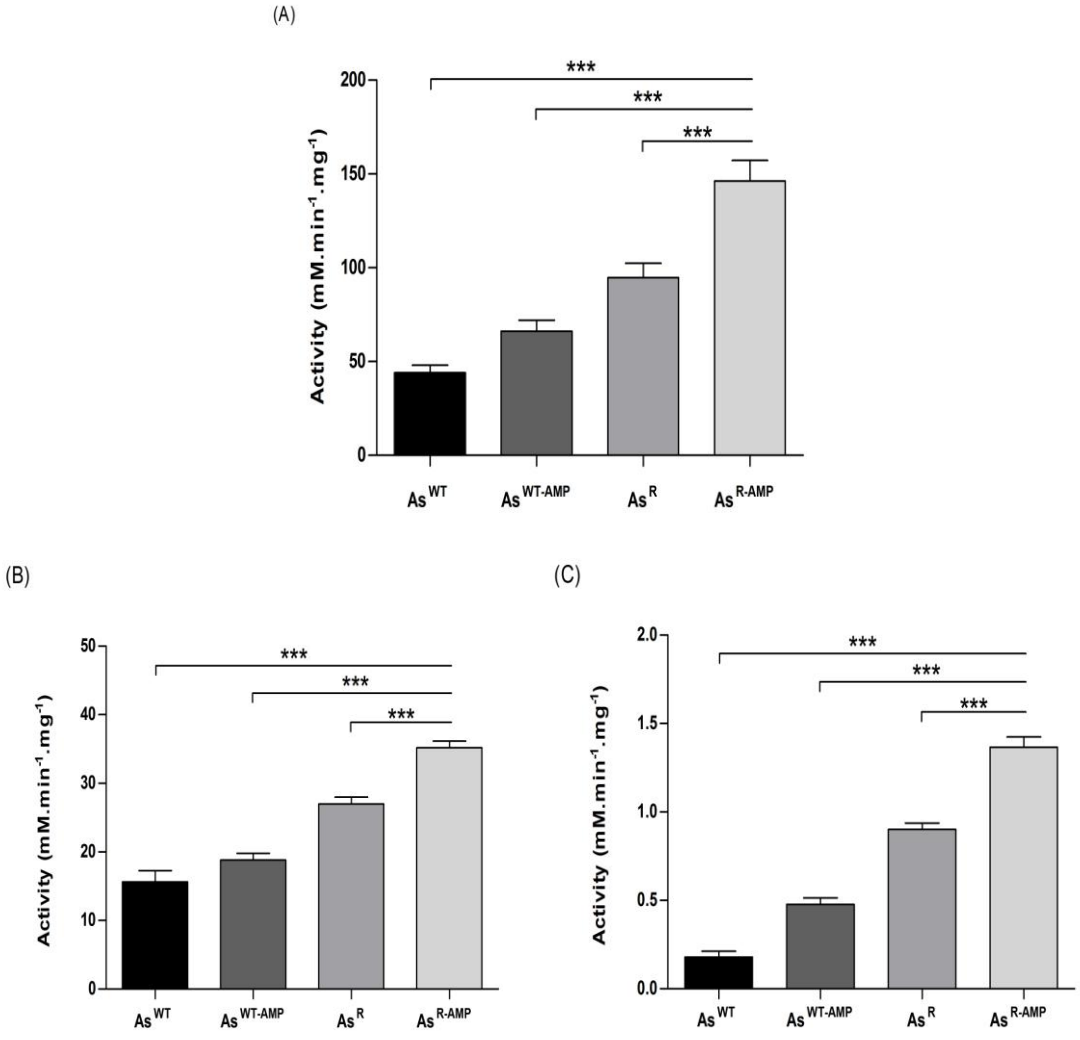

Figure 4. β-lactamase activities of the As^{WT} and As^{R} strains, having grown in the absence and presence of ampicillin. (A) β-lactamase activity against 100μM of ampicillin (B) β-lactamase activity against 100μM of cefotaxime (C) β-lactamase activity against 100μM of imipenem. Six independent experiments were performed and the arithmetic means $\pm$ the standard deviations were plotted. An *** indicate a ρ_{value} <0.0001 determined by one-way ANOVA.

2.4.3. Effect of subinhibitory concentrations of ampicillin on biofilm formation

Both *A. salmonicida* strains used in this study, As^{WT-AMP} and As^{R-AMP} show a great ability to form biofilms when they are grown in presence of ampicillin (Figure 5). These results demonstrate that the biofilm formation is a defense mechanism against antibiotics.

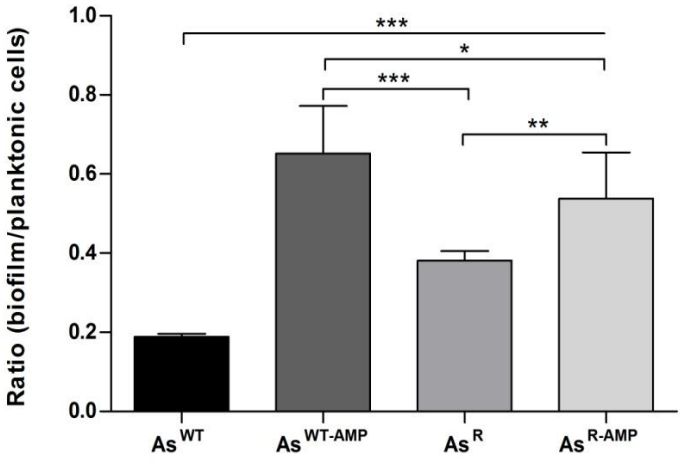

Figure 5. Ability to form biofilm of As^{WT} and As^{R} strains, are growing in the absence and presence of ampicillin. Three independent experiments were performed and the arithmetic means ± the standard deviations were plotted. A * indicate a ρ_{value} <0.05, ** indicate a ρ_{value} <0.001 and *** indicate a ρ_{value} <0.0001, determined by one-way ANOVA.

Biofilm is formed when bacterial cells attach to a surface and grow into a mass encapsulated by an exopolymer matrix. It helps microorganisms to present reduced susceptibility due the slow antimicrobial penetration, slow growth and protection of “persister” cells. These cells as mentioned before, are phenotypic variants that spontaneously switched from the wild type state and present antimicrobial tolerance (Roberts and Stewart 2005). However, the expression of biofilm phenotype in these cells occurs by the involvement of TA modules proteins, some of which has been described in *E. coli* and *P. aeruginosa* (Wang and Wood 2011). The TA modules are able to influence the c-di-GMP levels and this in turn regulates QS systems, which modulate genes expression linked to biofilm formation and others virulence factors (Kozlova et al. 2011).

In this way, the antibiotic stress induces biofilm formation, protecting persister cells that may easily develop an adaptive response to enhance survival under stress conditions.

2.4.4. Effect of subinhibitory concentrations of ampicillin on virulence factors

When *A. salmonicida* strains were grown in the presence of ampicillin, we did not observe significant changes in expression of virulence factors such as exotoxins. However, we observed that the basic phenotype of antibiotic susceptibility alter is related to their production. The resistant strains (As^{R} and $As^{R\text{-}AMP}$) showed a higher production of hemolysins and cytotoxins (Table 1 and Figure 6) when compared to the wild type strain (As^{WT}).

Table 1. Hemolytic and Proteolytic activities

Strains	Hemolytic activity [a]	Mean of protease activity ± SD (%)
As^{WT}	+	100 ± 3.6
$As^{WT\text{-}AMP}$	+	102.1 ± 3.6
As^{R}	++	72.9 ± 3.1[b,c]
$As^{R\text{-}AMP}$	++	77.1 ± 3.1[b,c]

[a] Hemolytic activity scored semiquantitatively: + α-hemolysis, ++ β-hemolysis.
[b] Differences between As^{WT} and As^{R} or $As^{R\text{-}AMP}$ were statistically significant (ρ_{value} <0.001), determined by one way ANOVA.
[c] Differences between $As^{WT\text{-}AMP}$ and As^{R} or $As^{R\text{-}AMP}$ were statistically significant (ρ_{value} <0.001), determined by one way ANOVA.

In *Aeromonas* spp. the expression of exotoxins is also associated with QS systems and these in turn are dependents of c-di-GMP levels. If, due to their different metabolisms, the resistant strain have different intracellular concentrations of c-di-GMP, when compared to the wild-type strain, differences in QS systems regulations can be expected. Consequently, expression of genes encoding virulence factors will be different between strains (Kozlova et al. 2011; Khajanchi et al. 2012).

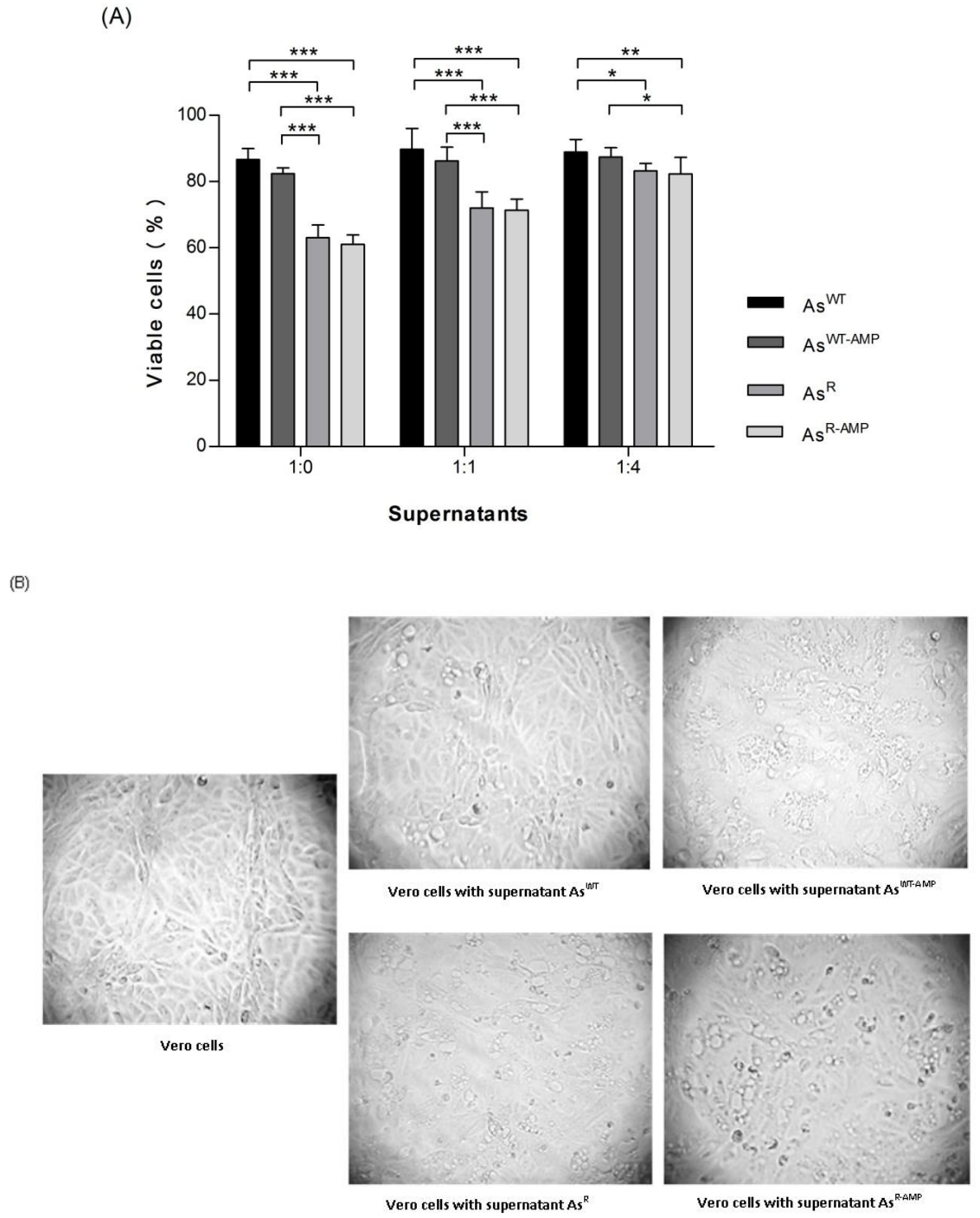

Figure 6.Cytotoxic effects in vero cells, after 48h of exposition - supernatants produced by As^{WT} and As^{R} strains grown in the absence and presence of ampicillin. (A) Three independent experiments were performed and the arithmetic means $\pm$ the standard deviations were plotted. An * indicate a ρ_{value} <0.05, ** indicate a ρ_{value} <0.001 and *** indicate a ρ_{value} <0.0001 determined by two-way ANOVA. (B) Evaluation of morphological aspects in Vero cells monolayers incubated with supernatants during 48h (supernatant non-diluted).

However, when we evaluate the production of proteases, a decrease on their production was apparent (Tabela 1). This result was not expected, because normally the mechanism that regulates proteases production is the same of hemolysins or cytotoxins production, as described for *A. hydrophila* in Khajanchi et al. (2012). However, another study in *A. salmonicida* showed that bacteria with lower susceptibility to antibiotics may present changes in the OM profile. Those changes result in lower membrane permeability that consequently leads to a restriction in proteases export, namely the major exoprotease (caseinase) (Griffiths and Lynch 1989). Probably, the loss of activity of this enzyme in our study explains the lower proteolytic activity observed in As^{R} and $As^{R\text{-}AMP}$.

In conclusion, in this work we demonstrated that the antibiotic exposure modifies the bacterial growth, induces β-lactamase production and promote biofilm formation in *A. salmonicida*. These processes seem to hold high importance for adaptation to new environments and conditions. When these strains are adapted (strain As^{R}), the intrinsic mechanisms of resistance keep activated as is the case of β-lactamases production, membrane permeability and other mechanisms. Virulence factors are expressed and their expression is dependent on QS systems which are regulated by c-di-GMP intracellular levels. This secondary cellular messenger in turn depends on bacterial metabolism. We may state that, although under complex regulatory networks, both the defense mechanisms and the virulence processes are closely related and of great relevance when one needs to prevent bacterial pathogenesis.

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CHAPTER III

STUDY II

3. COMPARATIVE PROTEOMICS OF *AEROMONAS SALMONICIDA* IN RESPONSE TO AMPICILLIN

3.1. ABSTRACT

This study consists on a large-scale proteomic experiment analyzing the response to β-lactams in *Aeromonas salmonicida* (As). We compared the intra and extracellular proteome of As wild-type strain (minimal inhibitory concentration [MIC] = 0.5µg/mL) against an *A. salmonicida* ampicillin resistant strain (MIC = 128µg/mL), derivate from the wild type strain. The technical approach used two dimensional gel electrophoresis followed by mass spectrometry. Changes in abundance were exhibited by 119 proteins of the intracellular proteome and by 53 proteins of the extracellular proteome. We observed an increase in β-lactamases production, a decrease in membrane permeability, overexpression of EPs and a possible involvement of OMV, therefore suggesting an activation of intrinsic mechanisms of antibiotic resistance. On the other hand we also observed an adaptive response consisting on growth rate reduction, alterations in metabolism with an increase in proteins biosynthesis, energy production and catabolism of carbohydrates. Evidences for increased oxidative stress and SOS response were also obtained. In conclusion, our data puts in evidence, for the first time, the systemic changes occurring in *A. salmonicida* in consequence of exposure to β-lactam antibiotics.

Key words: comparative proteomics, β-lactams, 2-DE, mass spectrometry, adaptive resistance

3.2. INTRODUCTION

Aeromonas species are mainly present in aquatic environments and they can infect humans (mainly, *Aeromonas hydrophila, Aeromonas caviae, Aeromonas veronii* / *Aeromonas sobria*) and animals (mainly *A. salmonicida* in fishes). The opportunistic infections caused by these species, are typically difficult to treat, because they present resistance to diverse groups of antibiotics. Resistance to penicillins, cephalosporins and carbapenems is commonly determined by chromosomally encoded β-lactamases, and resistance to tetracyclines, aminoglycosides, chloramphenicol and trimethoprim is usually associated with the presence of mobile genetic elements. Nevertheless, β-lactams antibiotics are the most frequently used for treatment (Janda and Abbot 2010; Carvalho et al. 2012). The inhibition mechanism of this kind of antibiotics is initiated by binding to cell wall transpeptidases (PBP's), blocking an important step in PG biosynthesis (Alvarez-Ortega et al. 2010).

Resistance in Gram negative bacteria can occur by several mechanisms, mainly drug inactivation by β-lactamases, drug extrusion through EPs, changes in OM permeability and PBP's modification (Alvarez-Ortega et al. 2010). In *Aeromonas* species, production of chromosomally encoded β-lactamases is the most important mechanism of resistance to β-lactams (Tayler et al. 2010).

At the present it is well established that besides the genes involved in the aforementioned mechanisms, several other genes exist that contributing to the process of β-lactams resistance (Vranakis et al. 2014). The antibiotic resistance is a result of bacterial adaptive success to an unfavourable environment (Händel et al. 2013). Bacterial stress responses rely on coordinated expression of genes that alter different cellular processes and act in concert to improve the bacterial tolerance (Renzone et al. 2005).

To identify molecules and regulatory networks involved in this adaptive response, proteomic technologies include the best tools available at the present, namely comparative proteomics (Radhouani et al. 2012). The comparative proteomics analysis consists in a protein separation step (two-dimensional electrophoresis is the most widely used), quantitative comparison

between expression profiles of proteins obtained from different biological states and identification of relevant proteins by mass spectrometry (MS) procedures (Renzone et al. 2005; Radhouani et al. 2012).

In this way, and in order to get more insights into the physiological changes upon antibiotic stress and bacterial adaptation in *Aeromonas* spp., we compared the subproteomes (intra and extracellular proteomes) of the wild-type strain *A. salmonicida* CECT894^{T} (As^{WT}) against laboratory derivated ampicillin resistant strain (As^{R}).

3.3. MATERIAL AND METHODS

3.3.1. *Aeromonas* strains

A. salmonicida CECT894^T was used as wild-type strain (As^{WT}). Its derived resistant strain (As^R) was previously obtained as described in chapter II, section 2.3.1. The MIC to ampicillin for two strains was determined as described in chapter II, section 2.3.2.

3.3.2. Measurement of growth rate

As^{WT} and As^R strains were cultured in LB and LB with ampicillin (128µg.mL^{-1}), respectively. The OD_{550nm} was adjusted to 0.05, corresponding approximately to 10^6 cells mL^{-1}. These suspensions were then incubated at 30ºC, during 24h with agitation at 180rpm. Throughout the growth aliquots were removed for subsequent reading of the OD at 550nm on a spectrophotometer UV-VIS (mini-1240, Shimadzu). This experiment was made in three independent assays and performed in triplicate.

3.3.3. Proteins Extraction

The proteins extracts were obtained simultaneously for As^{WT} and As^R. Cultures were grown to the late exponential phase (OD 0.9 at 550nm) in LB and LB with ampicillin (128µg.mL^{-1}), respectively. The cells were separated from the supernatant by centrifugation at 8000xg for 10min at 4ºC. The As^{WT} and As^R protein extractions were made in three independent experiments and the protein quantification was performed in triplicate.

3.3.3.1. Intracellular Proteins

The cell pellets were washed three times in 10mM phosphate buffered saline pH 7.4. After that they were resuspended in 1mL of lysis buffer solution [7M urea, 2M thiourea, 4% cholamidopropyl dimethylammonio-1-propanesulfonate (CHAPS), 30mM Tris base, pH 8.5]. Crude cell-free extracts were obtained by sonication in ice to minimize protein damage, during 2min, using a 30% duty

cycle, 2s pulses with intervening periods of 3s. The intracellular protein solution was incubated with 1mg.mL^{-1} of Dnase I (GE Healthcare, Sweden) and 10mM of protease inhibitor mix (GE Healthcare, Sweden) during 1h at 15ºC. The final solution was collected by centrifugation at 20000xg for 40min at 4ºC and then, the protein concentration was measured using the 2-D Quant Kit – GE Healthcare, following the manufacturer's instructions.

3.3.3.2. Extracellular Proteins

The supernatant was filtered through a 0.2µm filter and the proteins were extracted by precipitation at -20ºC (during at least 1h), with ice-cold 20% trichloroacetic acid in acetone containing 2% of β-mercaptoethanol. The samples were then centrifuged at 10000xg for 15min at 4ºC and the protein pellets were washed twice with ice cold acetone, followed by air-drying. The proteins were directly solubilized in lysis buffer solution and protein concentration was determined using the Bio-Rad *DC*™ Protein Assay Kit, following the manufacturer's instructions.

3.3.4. Intracellular Proteome

3.3.4.1. DIGE analysis

Samples were minimally labelled with G-Dyes (Refraction-2D™ labelling kit) according to the instructions of NH DyeAgnostics (Germany). To determine quantitative differences between the intracellular proteomes, the protein sample of the As^{WT} strain was labelled with G-Dye200 and the protein sample of the As^{R} strain with G-Dye300. A pooled internal standard representing equal amounts of both protein samples was labelled with G-Dye100. For each labelling reaction 25µg protein was incubated with 200pmol G-Dye for 30min. To stop the reaction, 1µL of stop solution was added and the mixture was incubated 10min. All labelling incubations were carried out on ice and protected from light.

3.3.4.2. Isoelectric focusing (IEF)

Samples labelled with each G-Dye were pooled (75µg of total protein) and adjust to a final volume of 350µL with rehydration buffer [7M urea, 2M thiourea, 2% CHAPS, 0.3% DTT and 2% carrier ampholytes (bio-lyte solution, pH 3-10)]. Samples were then applied to IPG strips (18cm, pH 3-10 NL; Bio-Rad Laboratories, USA) for passive rehydration during 10h at room temperature. Rehydrated strips were subjected to IEF program on a Protean IEF Cell (Bio-Rad Laboratories, USA): step of 150V for 1h, step of 500V for 2h, linear gradient of 1000V for 6h, linear gradient of 10000V for 3h, step of 40000Vh and final step of 500V for 10min. IPG strips were equilibrated twice for 15min in equilibration buffer [6M urea, 30%(w/v) glycerol, 2%(w/v) SDS in 50mM Tris-HCl buffer pH 8.8]. In the first equilibration step, 1% DTT was added to the original equilibration buffer and 4% iodoacetamide to the second step. Bromophenol blue was also added to both solutions.

3.3.4.3. SDS-PAGE

IPG strips were transferred to a 12.5% polyacrylamide gel and run in Protean Plus Dodeca Cell (Bio-Rad Laboratories, USA) connected to a cooling bath Model RM 6 Lauda (Lauda-Brinkmann, New Jersey) at 15ºC. Electrophoresis was performed in Tris/glycine/SDS buffer. Proteins were separated at 200V until the dye front reached the bottom of the gel.

3.3.4.4. Image Analysis

Images were acquired and pixel intensity was obtained using a Pharos FX Plus Molecular Imager (Bio-Rad Laboratories, USA) and differential gel analysis was performed using PDQuest Advanced v.8.0.1 software (Bio-Rad Laboratories, USA). The statistical analysis used for comparison between the two groups (As^{WT} and As^{R}) was determined by a Student's t-test ($\rho_{value} \leq 0.05$). Only the significantly different spots were retained for further investigation.

3.3.5. 2-D gel electrophoresis of the extracellular proteome

The extracellular proteins (75µg total protein) were subjected to isoelectric focusing as described previously by Ebanks et al. (2006), but using the IPG strips pH 3-11 NL, 13cm (GE Healthcare, Sweden). IEF was conducted at 20ºC with active rehydration (50µA, 12h), following the focusing program with initial step of 150V for 1h, other step of 500V for 2h, linear gradient of 1000V for 6h, linear gradient of 10000V for 3h, step of 40000Vh and last step of 500V for 10min in the IPGphor system (GE Healthcare, Sweden). For the second dimension was made in 12.5% polyacrylamide gel, with run for 14h at 4W/gel using a Protean II xi Cell (Bio-Rad Laboratories, USA). The gels were stained with Comassie Brilliant Blue G-250 (Amresco, USA) and then scanned with GS-800 Calibrated Densitometer (Bio-Rad Laboratories, USA). Image analysis was carried out using PDQuest Advanced v.8.0.1 software (Bio-Rad Laboratories, USA). The statistical analysis for comparisons between the two groups (As^{WT} and As^{R}) used a Mann-Whitney Signed Rank Test ($\rho_{value} \leq 0.05$). Only the significantly different spots were retained for further investigation.

3.3.6. Protein identification of differential spots by mass spectrometry

The spots of interest were excised and successively guanidinated, digested with trypsin and N-terminal sulfonated to enhance the *de novo* sequencing (Sergeant et al. 2005). The tryptic peptides were then analyzed by tandem mass spectrometry on a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystem, USA). For protein identification, an in-house MASCOT server v.2.1.0 (Matrix Science, UK) was used with a database containing the NCBI protein sequences of *Aeromonas* spp.. To confirm the results, data analysis proceeded to PEAKS® Studio 6.0 (Bioinformatics Solutions Inc., Waterloo, Canada) and *de novo* sequencing and protein identification performed (Zhang et al. 2012). The PEAKS search parameters encompassed fragment mass error tolerance of 0.3Da, carbamidomethylation (57.02) as fixed modifications and methionine, histidine and tryptophan oxidation (15.99) as variable modifications. In addition, manual interpretation of the spectra was performed to confirm the previous results and the similarity of the identified peptide sequences was searched with FASTS algorithm (standard settings search (matrix PAM 120)

against UniProtKB; ρ_{value} <0.05 scores were considered significant) (Mackey et al. 2001). The theoretical pI was predicted using Compute pI/Mw tool via ExPASy (Standing 2003).

3.3.7. Bioinformatics analysis

An integrated analysis of all proteins found with differential expression was performed with STRING version 9.1 (Franceschini et al. 2013). A pattern of protein-protein interactions was obtained. To identify biological pathways altered in As^R GO (gene ontology) categories were used as biological processes.

3.4. RESULTS

The development and selection of resistant bacteria confers metabolic costs which can be measured based on growth rate. A more prolonged lag phase is observed in the growth curve of the As^R strain (Figure 1), which can be an evidence of a decreased bacterial "fitness".

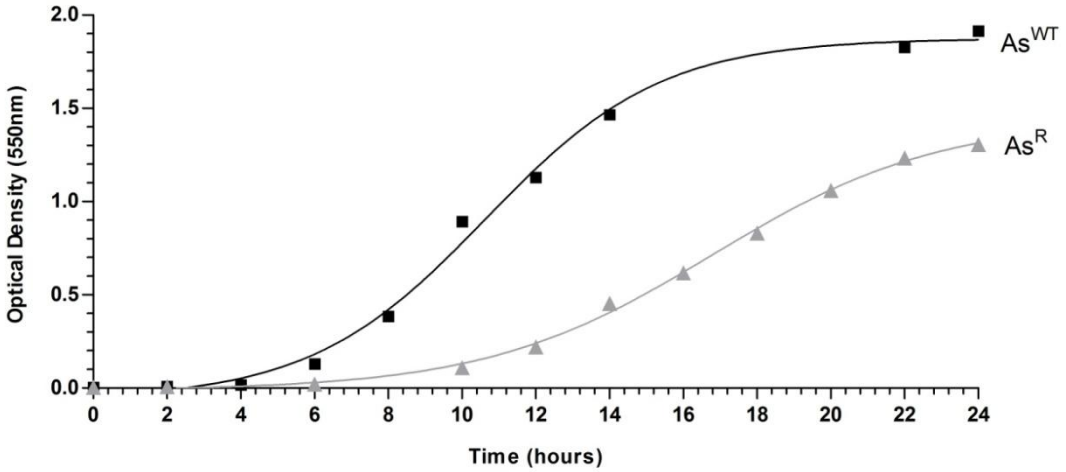

Figure 1. Growth patterns of *A. salmonicida* wild-type strain, As^{WT} and *A. salmonicida* derivated ampicillin resistant strain, As^R.

The physiological changes contributing for the resistance of the As^R strain are reflected on their proteome, thus we compared protein contents between As^R and As^{WT} strain. For intracellular proteome evaluation we used the DIGE technique and for extracellular proteome we used 2D-electrophoresis.

3.4.1. Intracellular Proteome

The intracellular proteome analysis revealed numerous changes in the protein abundance. Approximately 220 spots were visualized, 77 of which were overexpressed in As^R and 42 were underexpressed. A total of 50 spots were successfully identified by MS, representing 35 different proteins (Figure 2, Table 1 and 2). The intracellular proteins displaying an increased abundance in As^R are mainly involved in protein folding processes as is the case of molecular

chaperones and/or heat shock proteins (*grpE, htpG, groL, tuf1*), transcription and regulation processes (*hns, rpoA*), oxidative stress response (*ahpCF*), SOS response (*clpB*), nucleotide metabolism (*adk, purT, udp*), carbohydrate metabolism and energy production (*gap, pta, tkt, adhE, pckA, lpdA*), amino acid biosynthesis (cysk) and protein biosynthesis (*tsf, pros, pheS*) (Table 1). On the other hand, the proteins displaying decreased abundance are mainly involved in membrane transport (*ompAII, ompII, ompAI, tolC*), cell division (*tig*), protein biosynthesis (*fusA*) and glucose metabolism (*fbaA*) (Table 2).

3.4.2. Extracellular Proteome

The extracellular proteome analysis also revealed numerous changes in the protein abundance. In this case approximately 274 spots were visualized, 38 of which were overexpressed in As^R and 15 were underexpressed. A total of 21 spots were successfully identified by MS, representing 21 different proteins (Figure 3, Table 3 and 4). The extracellular proteins showing increased abundance in As^R are mainly involved in processing of proteins through molecular chaperones (*dnaK*), and in translation (*rpsA*), oxidative stress (*ahpC*), RNA degradation (*pnp*), carbohydrate metabolism and energy production (*prkA, gcvP, accC, gabD*), transport (*ASA_3341, napA, ASA_3982*) and antibiotic resistance (β-lactamase, *cphA5*) (Table 3). The extracellular proteins presenting decreased abundance in As^R are involved in membrane transport (*ompII, ompAI, ASA_4388*), transcription (*ASA_0555*), translation (*rplF*) and proteolysis (*ASA_3723*) (Table 4).

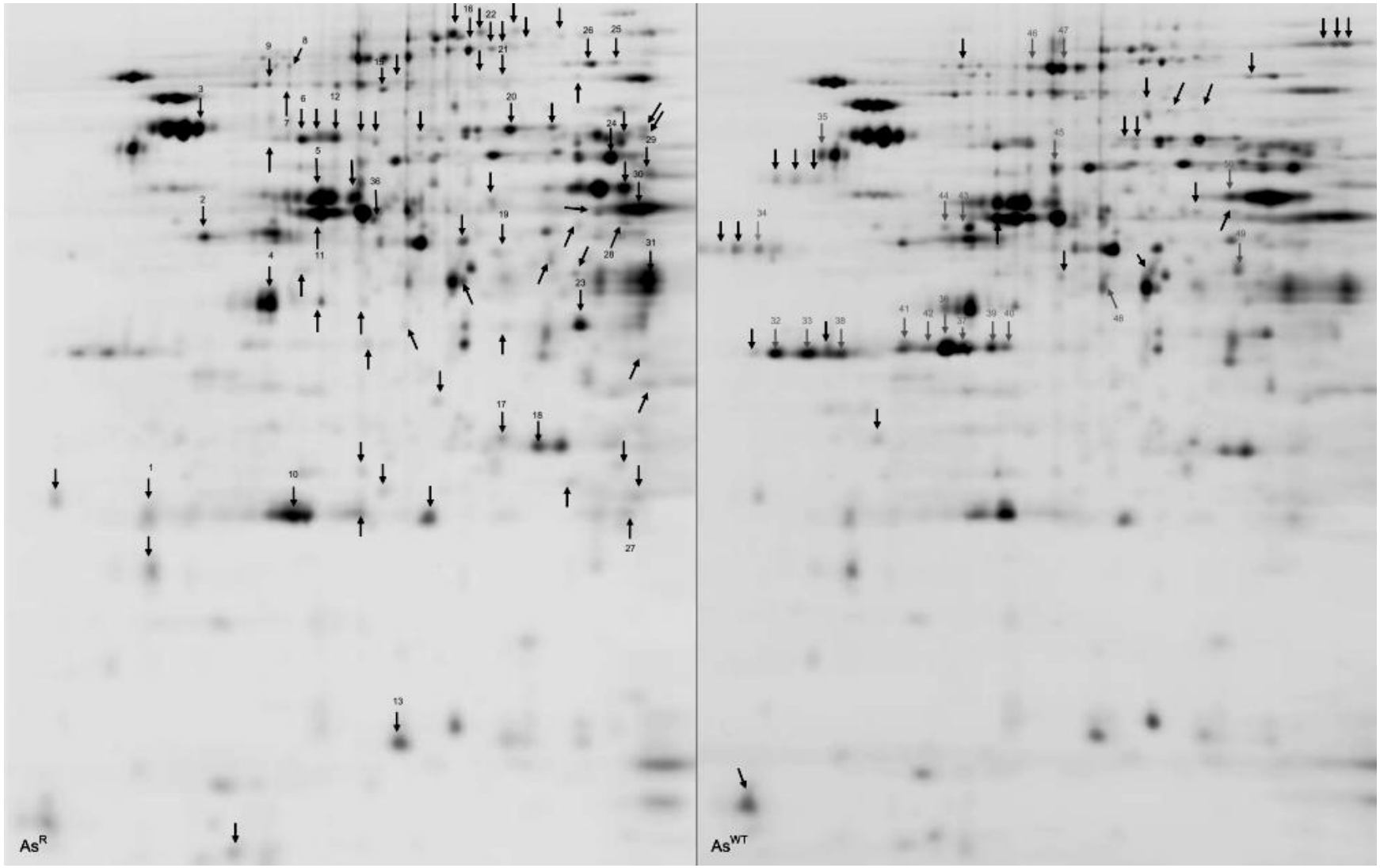

Figure 2. 2D-DIGE patterns of the intracellular proteins in wild type strain (As^{WT}) and derived resistant strain (As^{R}). The differential spots are indicated by arrows; the spots identified by mass spectrometry are numbered.

Table 1. Intracellular proteins identified by 2D-DIGE as being more abundant in As^R strain.

Biological Process[a] / Protein name[b]	Gi nr[b]	Gene name[b]	MW(Da)[c]	pI[c]	Spot nr[d]	Fold change[e]
Protein folding						
Protein GrpE	145299905	*grpE*	21,198.1	4.63	1	3.15
Chaperone protein HtpG	142851581	*htpG*	71,728.7	5.03	7/9	1.38/1.72
Elongation factor Tu	142850143	*tuf1*	43,299.6	5.17	11/13/30	1.50/2.23/ 2.09
Protein refolding						
60 KDa chaperonin	142853081	*groL*	57,320.0	4.87	3	1.47
Protein biosynthesis						
Elongation factor Ts	145300060	*tsf*	31,159.8	5.13	4	1.34
Proline--tRNA ligase	142850574	*proS*	62,722.6	5.35	15	2.08
Phenylalanine--tRNA ligase alpha subunit	142851709	*pheS*	37,303.4	5.59	19	2.87
Protein Processing						
Chaperone clpB	142850116	*clpB*	95,618.4	5.54	16/22	1.63/2.29
Aminoacid biosynthesis						
Cysteine synthase	142852737	*cysK*	34,305.5	5.82	23	2.23
Transcription						
DNA-directed RNA polymerase subunit alpha	142853682	*rpoA*	36,195.3	4.91	2	1.47
Transcription regulation						
DNA-binding protein	142852690	*hns*	15,506.6	5.72	13	1.53
Oxidative stress						
Alkyl hydroperoxide reductase subunit F	142852331	*ahpF*	56,176.0	5.24	6/12	2.10/2.23
Alkyl hydroperoxide reductase subunit C	142852330	*ahpC*	20,788.6	5.17	10	2.05
RNA degradation						
Polyribonucleotide nucleotidyltransferase	142850820	*pnp*	76,442.6	5.12	8	1.50
Purine metabolism						
Adenylate kinase	142851582	*adk*	23,196.5	5.62	17	1.85
Phosphoribosylglycinamide formyltransferase 2	142851925	*purT*	42,171.5	5.90	28	5.75
Pirimidine metabolism						
Uridine phosphorylase	142852591	*udp*	26,861.8	5.69	18	2.44
Carbohydrate metabolism						
Enolase	142853122	*eno*	45,736.0	5.18	5	1.77
Phosphoenolpyruvate carboxykinase [ATP]	142853411	*pckA*	59,795.7	5.65	20	1.18
Glyceraldehyde-3-phosphate dehydrogenase	142850592	*gap*	35,035.1	6.13	31	2.64
Transketolase	142850989	*tkt*	71,624.1	5.61	21	2.21
Dihydrolipoyl dehydrogenase	142850279	*lpdA*	50,500.2	5.86	24	1.55
Acetyl-CoA biosynthesis						
Phosphate acetyltransferase	142853056	*pta*	77,622.0	5.82	25/26	1.52/1.31
Alcohol metabolic process						
acetaldehyde dehydrogenase	142851455	*adhE*	96,348.7	6.13	27	Only As^R
Sodium ion transport						
Na(+)-translocating NADH quinone reductase subunit A	142852871	*nqrA*	48,791.2	6.13	29	2.10

[a] Biological processes are described in UniprotKB database; [b] Protein name, Gi-protein entry number and gene name in NCBI database – *A. salmonicida* strain A449; [c] Theoretical molecular weight (MW) in Dalton (Da) and isoelectric point of the protein determine in ExPASY program; [d] Spot numbering as indicate in figure 2; [e] The fold changes determined by PDQuest (ρ_{value} <0.05)

Table 2. Intracellular proteins identified by 2D-DIGE as being less abundant in As^R strain.

Biological Process[a] / Protein name[b]	Gi nr[b]	Gene name[b]	MW(Da)[c]	pI[c]	Spot nr[d]	Fold change[e]
Membrane transport						
Outer membrane protein	142851052	*ompAII*	35,157.7	4.91	32/33/38	2.14/3.46/ Only As^{WT}
Outer membrane porin II (OmpK40)	142851311	*ompII*	38,455.1	4.68	34	Only As^{WT}
Outer membrane protein	142851053	*ompAI*	35,807.1	5.33	36/37/39 /40/41/ 42	12.79/26. 07/30.18/ 47.29/ Only As^{WT}/ Only As^{WT}
Type I secretion outer membrane protein, TolC	142851431	*tolC*	49,536.2	5.24	43/44	2.75/ Only As^{WT}
Cell division						
Trigger factor	142851642	*tig*	47,875.5	4.82	35	1.69
Metabolic process						
Aspartate ammonia-lyase	142853085	*aspA*	52,544.7	5.41	45	1.49
Protein biosynthesis						
Elongation factor G	142850159	*fusA*	77,497.4	5.30	46/47	2.88/1.24
Glucose Metabolism						
Fructose-bisphosphate aldolase class II	142853151	*fbaA*	38,933.3	5.43	48	1.75
Aminoacid degradation						
Phospho-2-dehydro-3-deoxyheptonate aldolase	142851615	*aroG*	38,101.6	5.79	49	3.13
Arginine deiminase	142850093	*arcA*	45,539.8	5.88	50	2.45

[a] Biological processes are described in UniprotKB database; [b] Protein name, Gi-protein entry number and gene name in NCBI database – *A. salmonicida* strain A449; [c] Theoretical molecular weight (MW) in Dalton (Da) and isoelectric point of the protein determine in ExPASY program; [d] Spot numbering as indicate in figure 2; [e] The fold changes determined by PDQuest (ρ_{value} <0.05)

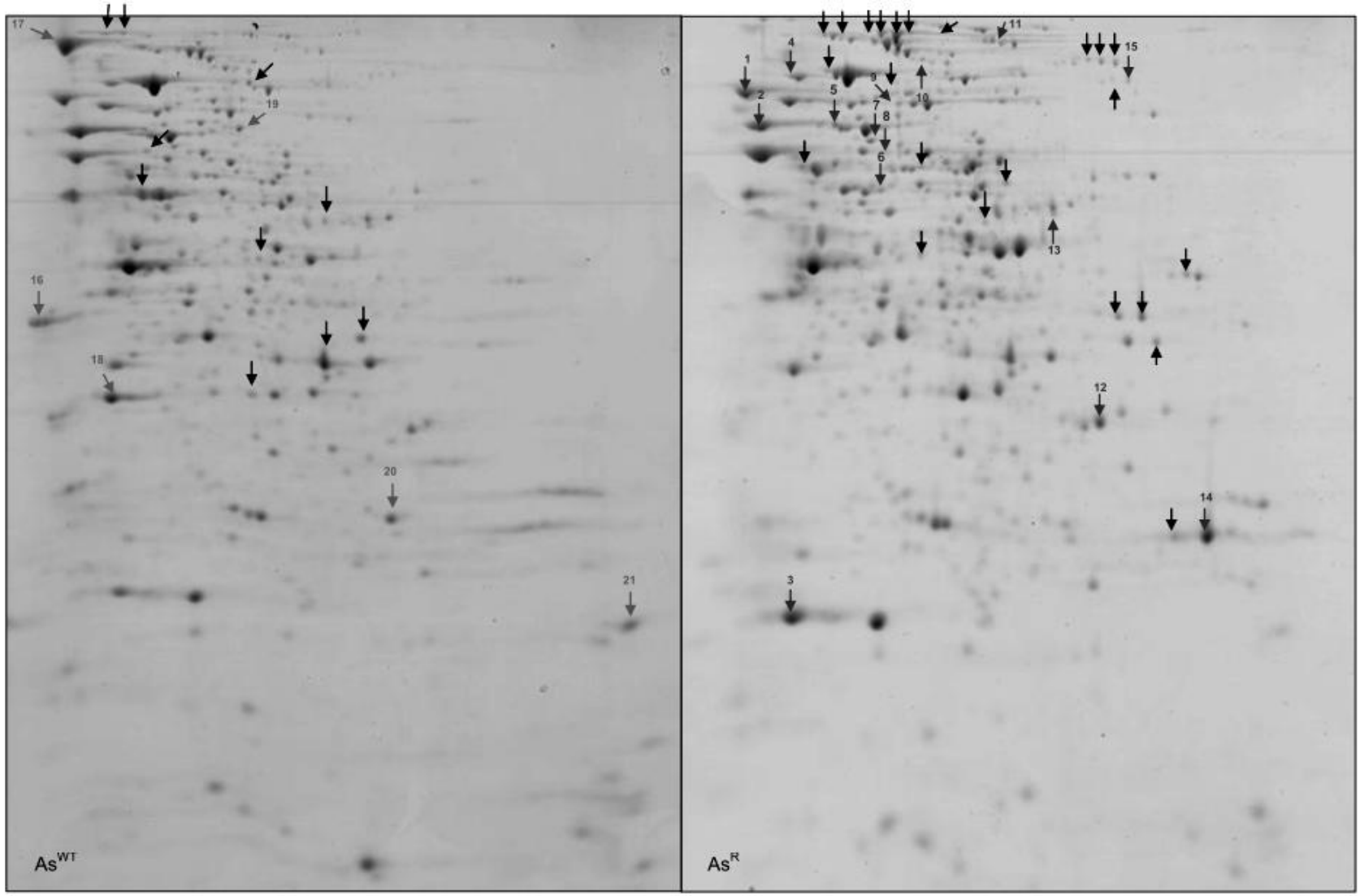

Figure 3. 2D electrophoresis analysis of the extracellular proteins in wild type strain (As^{WT}) and derived resistant strain (As^{R}). The differential spots are indicated by arrows, the spots identified by mass spectrometry are numbered.

Table 3. Extracellular proteins identified by 2D as being more abundant in As^R strain.

Biological Process[a] / Protein name[b]	Gi nr[b]	Gene name[b]	MW(Da)[c]	pI[c]	Spot nr[d]	Fold change[e]
Protein folding						
Chaperone protein DnaK	142852676	*dnaK*	69,557.4	4.79	1	3.92
Translation						
30S ribosomal protein S1	142851526	*rpsA*	61,401.4	4.91	2	4.84
Oxidative stress						
Alkyl hydroperoxide reductase subunit C	142852330	*ahpC*	20,788.6	5.17	3	9.46
RNA degradation						
Polyribonucleotide nucleotidyltransferase	142850820	*pnp*	76,442.6	5.12	4	4.75
Unknown						
Uncharacterized protein	142850943	*ASA_1143*	68,281.9	5.63	5	4.08
Aminobutyric acid catabolic process						
Succinate-semialdehyde dehydrogenase	142852700	*gabD*	51,590.2	5.42	6	12.11
Membrane transport						
ABC-type transporter, ATPase component	142852999	*ASA_3341*	62,400.7	5.38	7	7.48
Periplasmic nitrate reductase	142852468	*napA*	92,834.6	7.89	10	16.11
Protein biosynthesis						
Lysine--tRNA ligase	142850751	*lysS*	58,256.4	5.35	8	4.98
Metabolic process						
Serine protein kinase, PrkA	142852027	*prkA*	74,044.9	5.54	9	8.22
Glycine dehydrogenase (decarboxylating)	142852334	*gcvP*	103,494.4	5.98	11	48.08
Transport						
TRAP transporter solute receptor, TAXI family	142853611	*ASA_3982*	35,223.1	8.27	12	168.93
Acetyl-CoA metabolism						
Acetyl-CoA carboxylase, biotin carboxylase	142850774	*accC*	48,989.5	6.38	13	34.23
Antibiotic resistance						
Class B β-lactamase	142853252	*cphA5*	27,908.2	8.87	14	15,284.86
RNAr processing						
RNAr large subunit methyltransferase K/L	142851745	*rlmL*	81,226.9	7.30	15	Only As^{IND}

[a] Biological processes are described in UniprotKB database; [b] Protein name, Gi-protein entry number and gene name in NCBI database – *A. salmonicida* strain A449; [c] Theoretical molecular weight (MW) in Dalton (Da) and isoelectric point of the protein determine in ExPASY program; [d] Spot numbering as indicate in figure 3; [e] The fold changes determined by PDQuest (ρ_{value} <0.05)

Table 4. Extracellular proteins identified by 2D as being less abundant in As^R strain.

Biological Process[a] / Protein name[b]	Gi nr[b]	Gene name[b]	MW(Da)[c]	pI[c]	Spot nr[d]	Fold change[e]
Membrane transport						
Outer membrane porin II (OmpK40)	142851311	*ompII*	38,455.1	4.68	16	Only As^{WT}
Outer membrane protein	142851053	*ompAI*	35,807.1	5.33	18	821.37
ABC-type transporter, periplasmic binding protein	142853982	*ASA_4388*	27,916.9	7.77	20	299.28
Enzyme (proteolysis)						
Microbial collagenase	142853363	*ASA_3723*	103,484.6	4.83	17	17.47
Transcription						
Transcriptional regulator, LysR family	142850400	*ASA_0555*	34,427.7	6.46	19	26.70
Translation						
50S ribosomal protein L6	142853692	*rplF*	18,510.3	9.77	21	8.61

[a] Biological processes are described in UniprotKB database; [b] Protein name, Gi-protein entry number and gene name in NCBI database – *A. salmonicida* strain A449; [c] Theoretical molecular weight (MW) in Dalton (Da) and isoelectric point of the protein determine in ExPASY program; [d] Spot numbering as indicate in figure 3; [e] The fold changes determined by PDQuest (ρ_{value} <0.05)

3.5. DISCUSSION

Resistance to β-lactam antibiotics is widely reported in *Aeromonas* spp*.,* but there is a lack of knowledge on the mechanisms involved in cell response in the presence of antibiotics. The differentially expressed proteins identified in this study give us new insights on antibiotic resistance in *A. salmonicida* showing a systemic view of cell metabolism changes related with this condition.

3.5.1. β-lactamases production

We observed that ampicillin adaptation in *A. salmonicida* involves the production of β-lactamases, an increase in abundance of fifteen thousand folds of CphA5 protein was found. The chromosomally mediated inducible β-lactamases were recognized as the major intrinsic mechanism of antibiotic resistance in *Aeromonas* spp.: different β-lactamases as AmpS, CepS and ImiS also known as CphA can be simultaneously produced. The expression of their genes is coordinated by a common regulatory pathway, described as blrAB system (Alksne & Rasmussen 1997). However, the activation of this resistance mechanism is dependent on the presence of antibiotic. The β-lactam antibiotics bind to the PBPs, promoting changes in the synthesis/remodelling process of PG and the signals produced lead to the coordinated expression of different β-lactamases (Tayler et al. 2010).

3.5.2. Outer membrane proteins and efflux pumps

OMP (OmpII, OmpAI, OmpAII, and TolC) were found less abundant in ampicillin derivate resistant strain (As^R) suggesting changes in OM permeability. A decrease in abundance in OmpA slows down the diffusion of various types of solutes, including drugs, thus contributing to the intrinsic resistance. This relationship was found in *Pseudomonas aeruginosa*, *Escherichia coli* and other Gram negative bacteria (Angus et al. 1982; Masi & Pagès 2013; Nikaido 1998; Delcour 2009; Dupont et al. 2004). Furthermore, the decrease in abundance of OmpII (porin with 40KDa) was also previously described in *Acinetobacter baumannii* associated to an increase of imipenem resistance (Clark 1996). Also

associated membrane permeability changes, is the loss of the major exoprotease (microbial collagenase, ASA_3723), as previously described in *A. salmonicida.* A relationship between membrane permeability and restriction of protease export from periplasm has been previously suggested (Griffiths & Lynch 1989), detectable in this species mainly by the loss of the major exoprotease.

Proteins involved in transport as EPs (ASA-3341 and ASA_ 3982 genes) were more abundant in As^R. These ABC transporters are responsible for the transport of toxins and a wide range of antibiotics promoting the extrusion of noxious agents to the environment protecting the bacterial cell (Amaral et al. 2014). TolC proteins described an integrant part of some EPs (AcrAB-TolC) were unexpectedly less abundant in As^R. The lacking of EPs was described in *E. coli* and related to an increase of antibiotic susceptibility (Sulavik et al. 2001). However, recent experimental work in the same species showed that the loss of TolC channel is a result of the inner membrane stress, and allows the membrane protection and cell surveillance under stress conditions (Dhamdhere & Zgurskaya 2010). In this context the physiological role of this protein, needs to be better understood.

Overall, the decrease of the membrane permeability and changes in EPs expression contributes to β-lactam resistance in *A. salmonicida*.

3.5.3. The “fitness cost” and protein quality pathway

During the adaptive response to ampicillin we observed changes in bacterial growth. As^R presented a decreased growth rate, concordant with the lower abundance of proteins related to cellular division (*tig*), transcription (*rplF*) and translation (*fusA*). These responses occur mainly due to changes in membrane permeability that determine amino acid, fatty acid and phosphate starvation in the bacterial cell (Srivatsan & Wang 2008). These cells in *E. coli* are known as persisters and they develop a specific stress response to survive (Bokinsky et al. 2013; Hansen et al. 2008). The persistence development was indicated in our study by the overexpression of *hns* (global transcription regulator) and *clpB,* stress associated protein produced when MqsR toxin (specific Rnase) is

activated. This is a global regulator that controls protein expression, identified as TA module (Hong et al. 2012; Kim & Wood 2010). The stress response is evidenced by the overexpression of DnaK, GroL, HtpG, GrpE, Tuf1 proteins. These proteins play a key role in protein folding events and cooperate to maintain conformational proteome integrity under stressful conditions. The involvement of these proteins was also previously observed in *E. coli* and *A. baumanii* (Thomas & Baneyx 2000; Calloni et al. 2012; Cardoso et al. 2010).

3.5.4. Metabolic changes

The stringent response to survive under antibiotic stress in *A. salmonicida* is modulated by upregulation of the expression of the routes involved in nucleotides (*adk, purT* and *udp),* amino acid (*cysK*) and protein biosynthesis (*proS, pheS* and *lysS).* The protein biosynthesis is dependent of transcription (*rpoA),* elongation *(tsf)* and translation (*rpsA*) processes and therefore proteins related to these processes were also found as more abundant. Proteins involved in the glucose metabolism and in the energy production (*gap, pckA, eno, tkt* and *prkA*) were also more abundant. The higher expression of these genes indicates a metabolic switch, so, although speculative, it seems that global regulators of bacterial metabolism might being modulate the susceptibility to antibiotics, as already seen by Händel et al. 2013. In *P. aeruginosa* the CbrAB system, which regulates carbon and nitrogen utilization was mentioned as able to modulate susceptibility to antibiotics (Yeung et al. 2011).

3.5.5. Oxidative stress response and adaptive mutation

In As^R we observed an increase in oxidative stress, as evidenced by the overexpression of genes of the tricarboxylic acid cycle (TCA) (*pta, adhE, accC, gcvP, lpdA* and *gabD)* and the genes encoding enzymes that catalyze the cellular detoxification (*ahpF* and *ahpC*). β-lactam antibiotics cause DNA-damage inducing cellular death (Kohanski et al. 2007). The oxidative stress results of hydroxyl radical formation through oxidation process that occurs during respiratory electron transport chain and conversion of NADH (produced during TCA cycle) to NAD^+. The hydroxyl radicals produced by Fenton reaction

are extremely toxic and damage proteins, membranes and lipids (Kohanski et al. 2007). An increase in the abundance of these molecules activates the bacterial cell damage response system (SOS response). The SOS response includes molecular chaperones (DnaK and GroEL; Dwyer et al. 2009), which were found as more abundant in the As^R strain. Another way to respond to oxidative stress is by altering the metabolism from aerobic to anaerobic (Händel et al. 2013). We observed this partial change by the overexpression of nitrate reductase (*napA*).

During several years oxidative stress was considered as a mechanism triggered by antibiotics, leading to lethal damage. However, nowadays, it is recognized as an important mutagenesis mechanism, which is associated with adaptive response (contributing to antimicrobial resistance) (Blázquez 2003; Tenaillon et al. 2004; Kohanski et al. 2010). Therefore, we found increased abundance of proteins involved in this process and a higher abundance of proteins related to error correcting repair systems, as is the case of polyribonucleotide nucleotidyltransferase (*pnp*).

3.5.6. Outer membrane vesicles

During the exoproteome analysis of As^R we identified proteins that are usually intracellular. An example is the CphA5 protein usually present in the periplasm but here detected also in the exoproteome. This data suggests the possible production of OMV. In *P. aeruginosa* the presence of extracellular β-lactamases was correlated to their packaging into membrane vesicles (Ciofu et al. 2000). The OMV production plays a key role in bacterial defense against the effects of environmental stressors, namely those that act in cell envelope, as occurs for resistance to antibiotics. At the present it is know, the existence of a link between changes in OM-peptidoglycan and vesiculation. This was demonstrated in *E. coli*, *Salmonella* spp. and *Vibrio Cholera* (Sonntag et al. 1978; Deatherage et al. 2009; Song et al. 2008). OmpA is recognized protein of OM-peptidoglycan and its deletion or truncation was shown to induce vesiculation (Sonntag et al. 1978; Deatherage et al. 2009; Song et al. 2008). We also observed a downregulation in OmpA in As^R strain which strengthens

even more this possibility. In *Aeromonas* species, namely in *A. caviae Sch3,* OMV production has already been observed (Angeles-morales et al. 2012).

3.5.7. Protein-protein interactions

The protein-protein interactions allowed to group proteins according their biological function. The overexpressed proteins (38) were included in three main clusters. Cluster 1 includes proteins involved in stress response, oxidative stress and protein folding; cluster 2 contains proteins involved in energy production and carbohydrate metabolism; and cluster 3 includes proteins related to protein biosynthesis (Figure 4). The underexpressed proteins (14) were grouped in three main clusters. Cluster A comprises proteins related to outer membrane and transport proteins; cluster B contains proteins involved in amino acid degradation; and cluster C includes proteins involved in cell division and transcription (Figure 5). These predicted protein-protein interactions allowed inferring about the main metabolic changes occurring during the adaptive response of *A. salmonicida* to ampicillin.

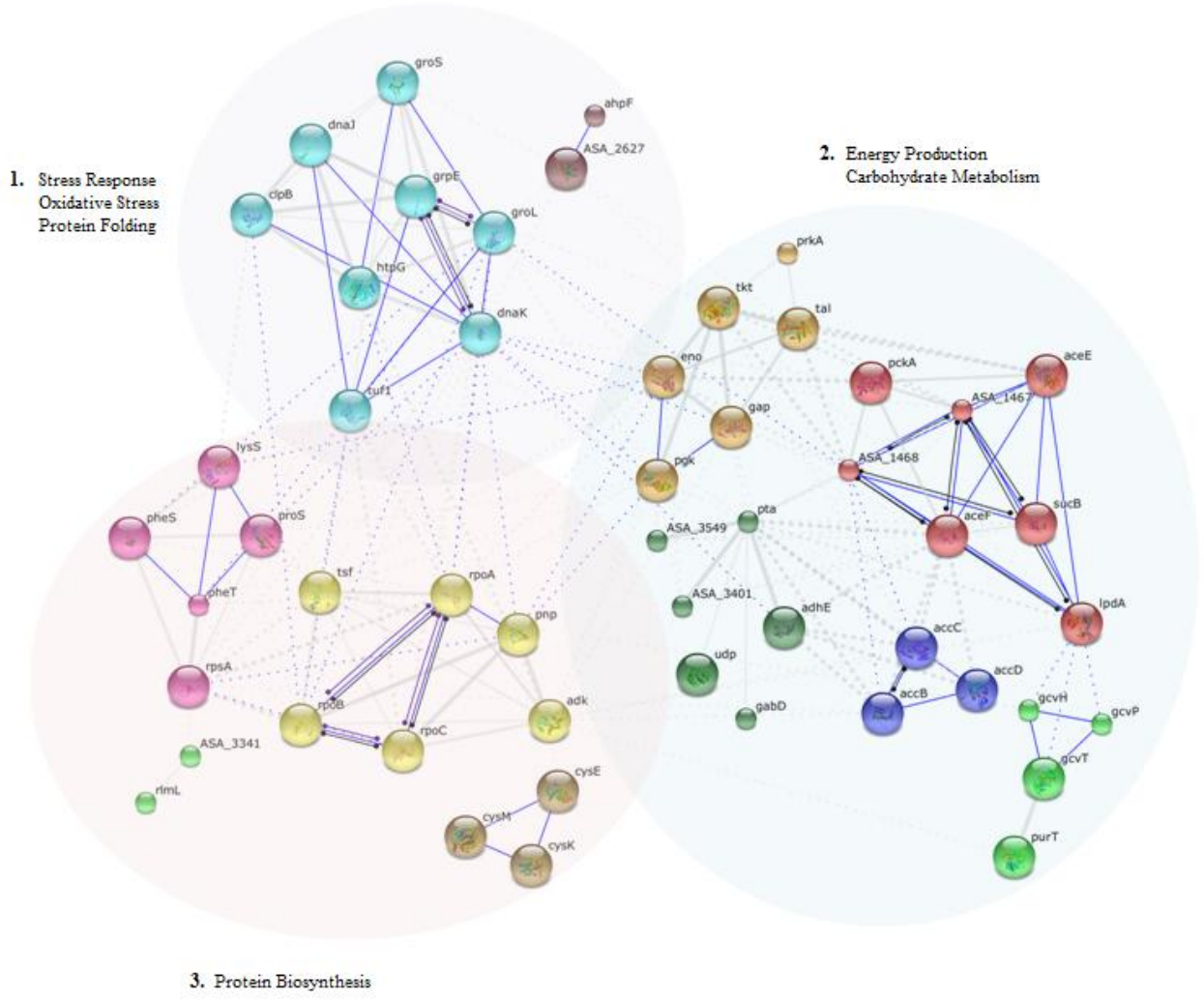

Figure 4. STRING protein network that integrate overexpressed proteins in As^R (38 proteins). Protein-protein interactions allow highlight three main clusters grouped according to the biological functions (89 interactions, ρ_{value} =0): 1. Proteins involved in stress response, oxidative stress and protein folding; 2. Proteins involved in energy production and carbohydrate metabolism; 3. Proteins related to protein biosynthesis.

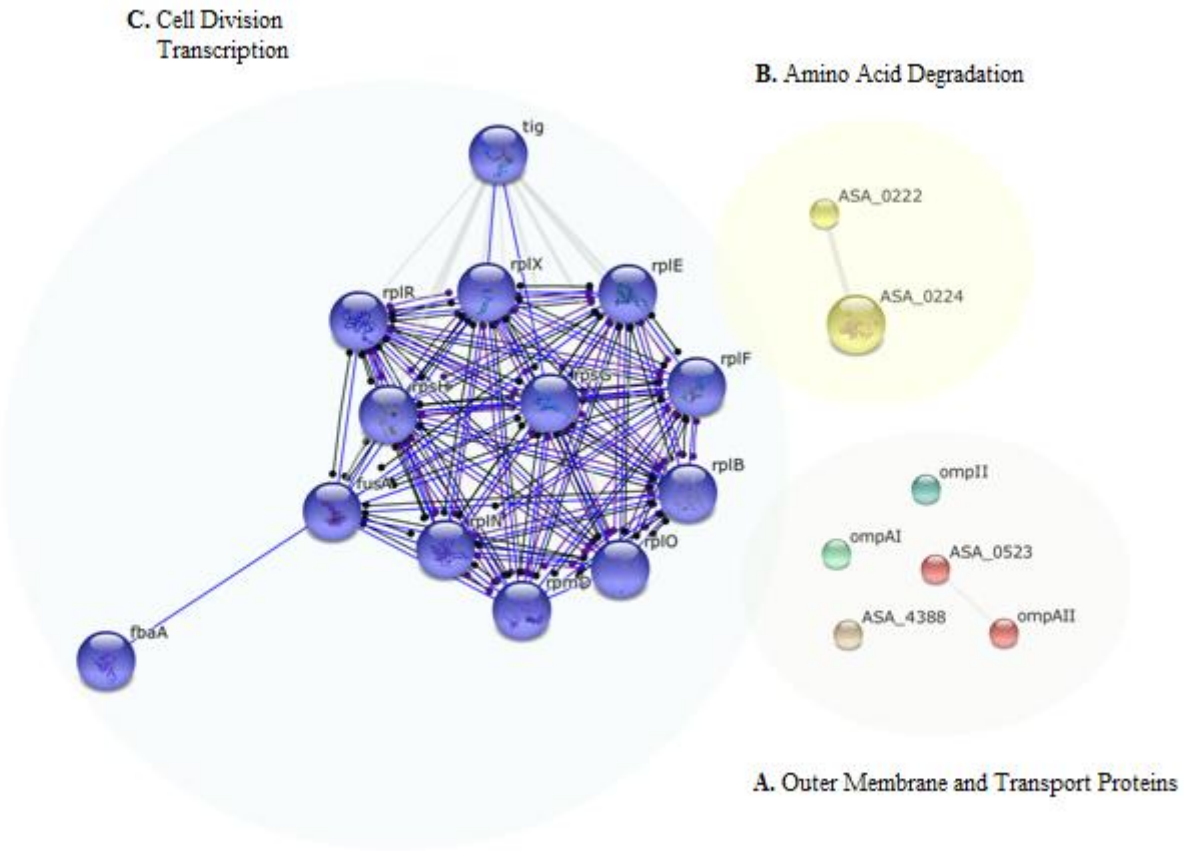

Figure 5. STRING protein network that integrate underexpressed proteins in As^R (14 proteins). Protein-Protein interactions allow highlight three main clusters grouped according to the biological functions (5 interactions, ρ_{value} =8.4e^{-3}): A. Proteins related to outer membrane and transport proteins; B. Proteins involved in amino acid degradation; C. Proteins involved in cell division and transcription.

3.6. CONCLUSION

Our study provides a first large-scale quantitative proteomic analysis of antibiotic response in *A. salmonicida* in an attempt to unravel the mechanism of β-lactamase induction. By using large-scale protein abundance profiling, multiple proteins of one pathway, as well as different pathways, can be studied together, providing a broad view on the different factors and possible interactions playing a role in the β-lactam response. In this sense, our results showed an adaptive response to ampicillin by *A. salmonicida* and allowed to identify metabolic changes. We observed an overexpression in proteins related to stress response, oxidative stress, protein folding, energy production, carbohydrate metabolism and protein biosynthesis and underexpression of proteins related to OM and transport, amino acid degradation, cell division and transcription. Hence, the adaptive response is dependent on the "persister" phenotype (reduced growth), SOS response (with eventual hypermutation) and probably involves the formation of outer membrane vesicles, as a defense mechanism. This study gives new and important knowledge related to β-lactam resistance of *Aeromonas* spp. and this knowledge may contribute to the design of strategies to prevent the development of infection processes and to the development of innovative therapeutic agents needed to treat opportunistic infections caused by *Aeromonas.*

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CHAPTER IV

GENERAL DISCUSSION

4. GENERAL DISCUSSION

Aeromonas spp. have emerged as opportunistic pathogens causing mainly intestinal infections in humans and furuncolosis in fishes and for which the main reservoirs are aquatic environments. This genus comprises strains that are able to produce a wide range of virulence factors. Additionally, these microorganisms often exhibit resistance to antibiotics and disinfectants, which represents a serious public health concern. Species of *Aeromonas* have chromosomally inducible β-lactamases that are recognized as the major mechanism of resistance to β-lactam antibiotics in these species. These antibiotics are frequently used in the treatment of infections and therefore have a high probability of failure when used to treat *Aeromonas*-associated disease (Janda & Abbott 2010; Parker & Shaw 2011; Dallaire-Dufresne et al. 2014).

In this study, *Aeromonas salmonicida* CECT894^{T} was used as the model organism. Repeated challenges with sub inhibitory concentrations of ampicillin promoted adaptive responses in bacteria, allowing to obtain an ampicillin resistant derived strain. The adaptation process of the resistant derived strain (As^{R}) involved changes in the phenotypic behaviour that are associated to changes in several metabolic networks.

Firstly, β-lactam antibiotics as is the case of ampicillin, have as cellular targets the PBPs, proteins with a crucial involvement in the biosynthesis of PG. If their function is inhibited, the remodelling process of PG stops. Chromosomally β-lactamases are induced by the *blrAB* system, as described by Tayler et al. (2010). In *Aeromonas* spp., three unrelated chromosomally encoded β-lactamases (AmpS, CepS, ImiS/CphA) can be simultaneously induced by the antibiotic exposure. This corroborates our observations of increased β-lactamase activity against ampicillin, cefotaxime and imipenem (in phenotypic evaluation), and overexpression of the CphA protein (a carbapenemase) in the proteomics analysis. Furthermore, we observed an increase of CphA levels, mainly in the extracellular proteome, although the protein is usually periplasmic. This suggests a possible involvement of OMV as demonstrated

in *Pseudomonas aeruginosa* (Ciofu et al. 2000), where extracellular β-lactamases are packaged into membrane vesicles, protecting the cells of the antibiotic action. It is worthy to mention that the production of OMV has been observed in *Aeromonas caviae* (Ciofu et al. 2000).

The production of OMV occurs when the PG remodelling stops or when membrane permeability changes (Kulp & Kuehn 2012). These two features were observed in the present study. A lower membrane permeability in As^R was also put in evidence by the decrease in proteins related to outer membrane, such as OmpA porins. These proteins allow the diffusion of hydrophilic antibiotics into the cell (Griffiths & Lynch 1989; Ebanks et al. 2005). In *A. salmonicida,* a change in membrane permeability is quickly detected by loss of the major exoprotease activity (caseinase) (Griffiths & Lynch 1989). This happens because OMP are involved in the export of certain enzymes. This may explain the lower protease activity and the decrease in the extracellular collagenase observed in As^R grown in the presence of ampicillin ($As^{R\text{-}AMP}$) comparing to that of As^R grown in an antibiotic-free culture.

Generally, changes of membrane permeability include down-regulation of porin synthesis and simultaneous overexpression of efflux pump components. The bacterial efflux transporters confer a general decrease of antibiotic susceptibility favouring the acquisition of additional mechanisms of resistance (Masi & Pagès 2013). In our study we observed an increase in abundance of ABC transporters, but a decrease in TolC protein. ABC transporters and TolC channel are involved in secretion of toxins and efflux of wide range of compounds namely antibiotics and they are regulated by *marA/soxS/rob* regulon (Masi & Pagès 2013). The TolC proteins allow the membrane protection and cell survival under stress conditions, as demonstrated by Dhamdhere & Zgurskaya (2010) in *Escherichia coli*. The loss of TolC proteins, although not expected, may result from the inner membrane stress that leads to metabolic shut-down and growth arrest.

When *A. salmonicida* is challenged by ampicillin, besides the increase of β-lactamases expression, we observed overexpression of EPs and lower permeability of membrane concomitantly with a set of changes in metabolic pathways.

The stress caused by exposure to antibiotic may stimulate the production of hydroxyl radicals, generated by the Fenton reaction through the bacterial metabolism such as the TCA cycle, the electron transport chain and the metabolism of iron (Martínez & Rojo 2011). These hydroxyl radicals could to contribute to cell death. This may be the reason why the proteomic approaches revealed, in As^R, increased abundance of AhpC and AhpF proteins related to hydroxyl radical synthesis, and of proteins related to TCA cycle like Pta, AdhE, AccC, GcvP, LpdA and GabD. Simultaneously, this stressful condition induces an SOS response which includes general stress response, heat-shock response and stringent response, all of which have impact on the regulation of error-prone polymerases (adaptive mutation) (Foster 2005).

The general stress response is observed when bacteria reduce the growth rate, with a set of genes being induced to help the cell to survive (Foster 2005). Therefore, the decrease of growth rate in A^R was accompanied by an increase of RpoA protein and a simultaneous decrease of the Tig protein. The Tig protein is able to promote the stop of growth and cell division. This dormant state is a reversible phenomenon and is dependent of a module of toxin-antitoxin proteins produced during stressful conditions, as referred for *E. coli* (Kim & Wood 2010). Generally, the toxin is a protein that inhibits an important cellular function such as translation and replication forming an inactive complex with the antitoxin. The toxin is stable and the antitoxin is degradable (Lewis 2010).

This dormant state is described in literature as being able to promote biofilm formation, contributing to drug resistance. Biofilms apparently serve as a protective habitat for persister cells (Kaldalu et al. 2004). In our study we observed $As^{R\text{-}AMP}$ with greater ability to form biofilm. Other studies in *Aeromonas* spp. also showed that aggregation or biofilm formation promote survival to antibiotics (Kaldalu et al. 2004).

Associated with “persister” formation and survival some studies in *E. coli* showed that ATP generation through the electron transport chain is an important process (Ma et al. 2010). Hence, we observed increased levels of Gap, PckA, Tkt and eno proteins in As^R strain. In turn, the expression of catabolic pathways involved in carbon and nitrogen utilization in *P. aeruginosa*

and *E. coli* has been demonstrated to be involved in metabolic regulation of the virulence factors like secretion systems, biofilm formation and swarming (Yeung et al. 2011; Raivio et al. 2013). In *A. salmonicida* we observed increased expression of hemolysins and cytotoxins. This may be due to the different levels of ATP that consequently alter cellular levels of c-di-GMP which in turn regulates the QS systems and therefore the genes involved in virulence, as has been shown in *Aeromonas hydrophila* (Kozlova et al. 2011).

The stress response also includes changes in the transcription process, in amino acids and proteins. Hence, we found an increase in abundance of proteins related with these processes, such as RpsA, Tsf, CysK, ProS and PheS. Simultaneously, the higher levels of oxidative stress induce overexpression of several molecular chaperones that help many proteins to reach a proper conformation or cellular location, and that participate in the reactivation or degradation of damage/unfolded proteins (Thomas & Baneyx 2000; Cardoso et al. 2010). For this reason we observed an increase in abundance of proteins like ClpB, HtpG, DnaK, GrpE and GroL.

All of the mentioned processes have impact in the regulation of error-prone polymerases and consequently mutagenesis, as mentioned in Foster (2005). Hence, in our study we found an increase of the polyribonucleotide nucleotidyltransferase, which is involved in DNA error repair and is responsible for the control of adaptive mutation, a process that generates genetic diversity, giving rise to phenotypes that are more adapted to survival under stress conditions.

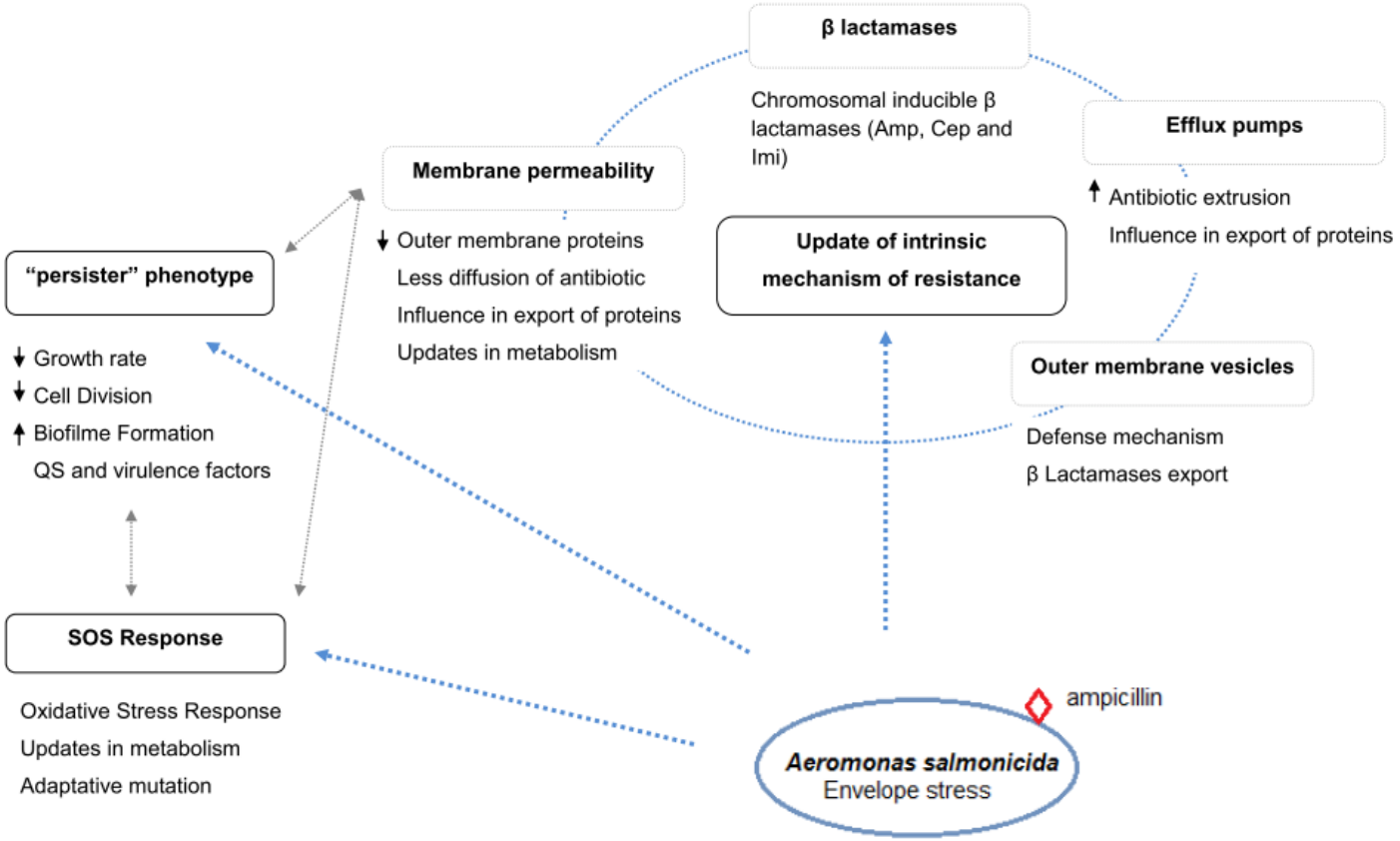

Figure 1. Adaptive response of *Aeromonas salmonicida* when challenged by ampicillin (β-lactam antibiotic): global view.

CHAPTER V

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

5. GENERAL CONCLUSIONS

The aim of this work was to characterize the response of *A. salmonicida* to an ampicillin challenge unravelling the complexity of resistance mechanisms of the β-lactam "resistome".

Firstly, we produced an ampicillin resistant strain from the wild-type *A. salmonicida* CECT894^T, which is sensitive to ampicillin. The resistant strain was obtained through gradual exposure to the antibiotic. Both strains were phenotypically characterized after being cultured in the presence and absence of antibiotic.

During the challenge with ampicillin (subinhibitory concentration) *A. salmonicida* strains showed changes in growth rate, β-lactamases production and biofilm formation directly associated with the presence of antibiotic. These processes are known to be important to respond to an antibiotic induced stress. A lower growth rate associated to a "persister" phenotype, increase the ability to form biofilm. These two processes associated with β-lactamases production allow the strain to develop antibiotic resistance. The resistant strain showed higher levels of production of virulence factors, like hemolysins and cytotoxins. Hence, antibiotic resistance and virulence are closely related processes simultaneously conditioning the development of infections and its treatment.

Antibiotic resistance results from important metabolic changes that are clearly observed when the *A. salmonicida* wild-type strain is compared by proteomic approaches to its resistant strain. Hence the gain of resistance is dependent of an adaptive response. In this response we observed important changes in intrinsic mechanisms of resistance associated with metabolic costs.

The main intrinsic mechanism of resistance triggered was the activation of the chromosomal inducible β-lactamases, showing an important involvement in β-lactam resistance in *Aeromonas* spp.. Then, loss of membrane permeability promoting less diffusion of antibiotic into bacterial cell and overexpression in EPs related to major extrusion of antibiotic. The results also suggested the

possible production of membrane vesicles a defense mechanism, being probably also involved in cellular communication.

Furthermore, the presence of antibiotic promotes reactive oxygen species production which in turn leads to SOS response. SOS response combines phenotype of persistence (decrease in growth rate and cell division) and oxidative stress response leading to a possible adaptation process through controlled mutations.

5.1. Future perspectives

As future work we believe it is important to confirm the production of OMV and, if the confirmation is positive, to characterize their content in order to describe their role and involvement in β-lactam resistance.

Evaluation of the phosphoproteome of the cell envelope and intracellular content is also an important issue in order to decipher the possible protein-protein interactions between phosphorylated proteins.

A comparative genomic analysis, addressing the genomes of the wild type and its resistant strain, is necessary to reveal whether or not some mutations have been fixed in the new strain, determining the stability of the resistance phenotype.

CHAPTER VI

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6. REFERENCES

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